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Cyanate Formation Following 2-Chloroacrylonitrile Exposure and the Role of Cytochrome P-450

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CYANATE FORMATION FOLLOWING 2-CHLOROACRYLONITRILE EXPOSURE AND THE ROLE OF CYTOCHROME P-450

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By

Paul Malichky

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Approved March 16, 2011

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ABSTRACT

CYANATE FORMATION FOLLOWING 2-CHLOROACRYLONITRILE EXPOSURE AND THE ROLE OF CYTOCHROME P-450

By

Paul Malichky

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Dissertation supervised by David A. Johnson, Ph.D.

2-Chloroacrylonitrile (2-CAN) is a volatile, toxic chemical used as a raw material to manufacture pesticides. Although the toxic mechanism is not fully understood, the hypothesis in the literature is that 2-CAN may be metabolized to cyanide. An understanding of the mechanism of 2-CAN toxicity will permit more appropriate treatments in the event of an accidental exposure.

Four groups of male Sprague-Dawley rats were given intraperitoneal (i.p.) injections. Group 1 was a control that received only saline. Group 2 received 40 mg/kg of 2-CAN. Group 3 received 100 mg/kg of 5-phenyl-1-pentyne (5P1P) an inhibitor of cytochrome P-450. Group 4 received 100 mg/kg of 5P1P one hour before 40 mg/kg of 2-CAN. Three hours following administration, the animals were euthanized via cardiac puncture. Cardiac blood was collected for cyanide and cyanate analysis. Liver and lung

tissues were harvested and analyzed for glutathione. In a separate experiment, the time to onset of hind limb paralysis was measured in animals receiving 40 mg/kg 2-CAN and 100 mg/kg 5P1P one hour prior to 40 mg/kg 2-CAN.

Blood cyanide levels were increased in the 2-CAN group, but did not exceed 0.06 μg/mL, a concentration well below the minimum for producing adverse health effects. Compared to control animals, the 2-CAN treatment group had depleted glutathione by 20% in the lung and 44% in the liver. When 5P1P was administered prior to 2-CAN, there was no significant change in glutathione concentrations.

Plasma cyanate levels in the 2-CAN group were 436 nmol/L plasma, a 390% increase over control. When a 5P1P was administered prior to 2-CAN, cyanate levels were reduced by 55% to less than 195 nmol/L plasma. In addition to decreasing plasma cyanate levels, 5P1P 1 hour prior to 2-CAN delayed the onset of hind limb paralysis from 6 minutes to 16 minutes.

Since cyanide was not generated in substantial concentrations following 2-CAN administration, the use of an antidote specific for cyanide for 2-CAN toxicity may not be effective. The findings of increased cyanate levels in 2-CAN, combined with the hind-limb paralysis data suggest that cyanate, not cyanide, is the toxic metabolic product resulting from acute 2-CAN exposure.

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LIST OF ABBREVIATIONS

2-CAN (2-Chloroacrylonitrile)

5P1P (5-phenyl-1-pentyne)

AN (Acrylonitrile)

ANOVA (analysis of variance)

AUC (Area Under Curve)

BPDS (Bathophenanthroline disulfonic acid)

b (intercept)

C (Celsius)

cc (cubic centimeter)

CEO (2-cyanoethylene oxide)

CN (cyanide)

CNO (cyanate)

CYP (Cytochrome P-450)

CYP2E1 (Cytochrome P-450 2E1)

CYP2F2 (Cytochrome P-450 2F2)

F (Fahrenheit)

FDNB (fluorodinitrobenzene)

g (gram)

GSH (reduced glutathione)

GSGG (oxidized glutathione)

HPLC (High performance liquid chromatography)

IAA (Iodoacetic acid)

i.p. (intraperitoneal)

kg (kilogram)

L (liter)

LC_{50} (Lethal Concentration in 50% of the animals)

LD_{50} (Lethal Dose in 50% of the animals)

MAN (Methacrylonitrile)

mbar (millibar)

m (slope)

mg (milligram)

MSDS (Material Safety Data Sheet)

NAC (n-acetyl-l-cysteine)

nm (nanomolar)

pH

ppm (parts per millon)

RCF (relative centrifugal force)

RD_{50} (Respiratory Depression of 50%)

rpm (revolutions per minute)

s.c. (subcutaneous)

s.e. (standard error)

µg (microgram)

µmol (micromol)

UV/Vis (Ultraviolet light in the visible spectrum)

I. Introduction

A. Statement of the Problem

2-Chloroacrylontrile (2-CAN) is an industrial chemical used in the manufacture of pesticides.[1] It is also used in the manufacturing process of norepinephrine reuptake inhibitors.[2]

An acute inhalation study indicated the LC_{50} value in rats was 28 ppm for a 4 hour exposure.[3] The LC_{50} is the airborne concentration in which 50% of the animals die. An LC_{50} of 28 ppm is classified as highly toxic.[4] 2-CAN is also toxic via the oral and dermal routes. The oral LD_{50} in rats is 32-78 mg/kg, the dermal LD_{50} is approximately 200 mg/kg in rats and less than 220 mg/kg in the rabbit.[5] In addition to lethality, the compound is also highly irritating[5] and a severe respiratory irritant. The RD_{50} is the concentration for which there is a 50% decrease in respiratory rate and is an indictor of sensory irritation. The RD_{50} value for 2-CAN is 5.7 ppm.[6]

In addition to being highly toxic, 2-CAN is also highly volatile with a vapor pressure of 85.4 mbar at 25°C.[5] For comparison, this makes 2-CAN slightly more volatile than ethanol which has a vapor pressure of 79.1 mbar at 25°C.[7] Although the compound is used as an industrial intermediate in a closed process, it should be considered a high risk chemical due to the high toxicity and high exposure potential. If there were a transportation incident or release at a production facility, it would be critical to know how to best treat people that were exposed.

Although it is known that 2-CAN is highly toxic, the mechanism of toxicity is not well understood. In cases of human exposure, the manufacturer's Material Safety Data Sheet (MSDS) recommends treatment for cyanide toxicity.[5] The traditional treatment for cyanide toxicity is sodium nitrite combined with thiosulfate.[8] In this treatment, hemoglobin is oxidized to methemoglobin which then scavenges cyanide.[9] Although it may be effective in saving a person's life in an emergency cyanide poisoning situation, this antidote is also a very risky treatment because methemoglobin reduces the body's ability to carry oxygen to the cells.[10]

An unpublished study by the manufacturer (Bayer) exposed four beagle dogs to 2-CAN at 97 mg/m3 for one hour. There was little effect on the dogs with no fatalities. There were no abnormalities in arterial blood gases and there were no abnormal findings in the lungs. However, microscopic examination of lung tissue did indicate evidence of inflammation and focal pulmonary edema. There was also a slight increase in plasma thiocyanate. This could suggest liberation of cyanide, but the increases were not statistically significant or toxicologically relevant.[11]

The hypothesis to be tested in this research dissertation is that the neurotoxicity of 2-CAN is primarily the consequence of the metabolite cyanate not cyanide.

B. Literature Survey

1. Compounds chemically similar to 2-CAN

A review of the literature suggests there is little known mechanistically about 2-CAN toxicity, but there is information that is known about other compounds similar to 2-CAN. The most common nitrile compounds are acrylonitrile (AN) and methacrylonitrile (MAN). **Figure 1** shows the structural similarities.

Figure 1. Structure of Acrylonitrile (AN), Methacrylonitrile (MAN), and 2-chloroacrylonitrile (2-CAN)

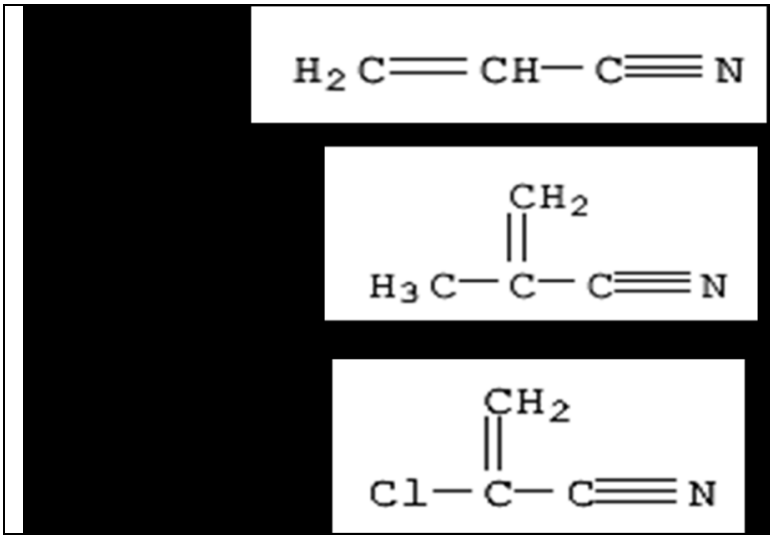


Figure 2 shows the relative toxicity of the nitriles. 2-CAN is more toxic than AN. AN is more toxic than MAN. (Figure 2)

Figure 2 – LD_{50} of AN, MAN, 2-CAN

Chemical	**Oral LD50 (rat)**	**i.p. LD50 (rat)**
Acrylonitrile	78 mg/kg[12]	65 mg/kg
Methacrylonitrile	200 mg/kg[13]	
2-Chloroacrylonitrile	32-72 mg/kg[5]	14-20 mg/kg[14]

Acrylonitrile (AN)

Billions of pounds of AN are produced annually.[15] It is used for the production of plastics and resins.[15] Acrylonitrile is a known rat carcinogen and suspected human carcinogen.[15]

There are two major metabolic pathways for acrylonitrile in the rat. The first is conjugation with reduced glutathione (GSH).[16] The AN-GSH conjugate is then processed in the mercapturic acid pathway and excreted in the urine.[17 18 19] More than 50% of AN is excreted via the urine[20] and less than 11% in the expired air.[21] The second metabolic pathway is cytochrome P450-mediated oxidation to the AN-epoxide 2-cyanoethylene oxide (CEO).[16 22 23] CEO is considered to be the carcinogen derived from AN.[24] Some of the epoxide is then further conjugated with glutathione and excreted via the mercapturic pathway and ultimately eliminated via the urine.[20]

Acrylonitrile is known to cause glutathione depletion.[25] Glutathione protects cells from AN toxicity until it is depleted. Thereafter, AN covalently binds to proteins in the cell.[26] One treatment for AN exposure is thiol-containing compounds such as n-acetyl-l-cysteine (NAC). The thiol can directly react with AN and prevent it from reacting with critical sulfhydryl groups in the body.[27] The thiol-AN conjugate would then be excreted in the urine via the mercapturic acid pathway. A second benefit of the thiol compound is as a precursor for the synthesis of GSH depleted by AN.[27] Treatment with GSH is not effective since GSH does not cross the cell membrane. NAC has been proven to be effecting in replenishing GSH levels and reducing toxicity in acetaminophen overdoses.[28] [29] A third benefit of the thiol is as a sulfur source for cyanide metabolism. The normal metabolism of cyanide involves the formation of thiosulfate. When glutathione levels in rats were depleted, there was an increased amount of AN binding to tissues and an increase by 300% in thiocyanate, a metabolite from the epoxide mechanism.[30] To form thiosulfate, a sulfur donor is needed for the rhodanese enzyme. Cysteine and its analogs can act as a sulfur donor[31] and decrease the amount of free cyanide.[27] Treatment of AN with cysteine and its n-acetyl derivates does not change the amount of non-oxidative mercapturic acid metabolites, but does decrease blood cyanide levels and the overall toxicity of AN.[27] [32] For these reasons, the German government recommends high intravenous doses of NAC as a treatment for accidental overexposure to AN by workers.[33]

Acrylonitrile leads to the generation of cyanide[26] [27], but acrylonitrile must first be metabolized by the cytochrome P450 oxidase CYP2E1 to the epoxide before cyanide is generated.[34] With CYP2E1 induction, cyanide levels increase in a dose dependent[35]

fashion, and following CYP2E1 inhibition cyanide levels decrease.[34 36] Induction of CYP2E1 increased cyanide levels and caused lethality at a dose not normally fatal.[36] Cyanide released from AN is considered to be a major contributing factor in AN-induced lethality[37] and human intoxication from AN exposure has been successfully treated with cyanide antidotes.[38] The conversion of AN to cyanide is also route dependent. The slower the rate of absorption, the more cyanide is generated from the same dose (intravenous<subcutaneous<intraperitoneal<oral).[39] The addition of glutathione or cysteine decrease the amount of cyanide generated from AN administration.[40]

Recent studies indicate CYP 2E1 is the specific CYP involved in the formation of the epoxide. [20 34 35 41 42] When rats were pre-treated with a CYP inhibitor, the amount of AN excreted in the urine decreased, and the total retention of AN in the animal increased.[21] Following administration of AN in CYP2E1-null mice, the cyanide levels were nearly undetectable and did not increase when the dose of AN was increased.[35 43] Following 40 mg/kg AN administered via gavage, all wild-type mice died within 3 hours while CYP2E1-null mice were able to survive 60 mg/kg/day for 5 day/week for 6 weeks.[35] When mice were treated with a CYP2E1 inhibitor or CYP2E1 null mice or were administered AN, there were no metabolites derived from the epoxide.[41] When CYP2E1 activity is induced by repeated acetone administration, there was an increase in mortality and lung damage following a single dose of AN.[44] Sub-chronic inhalation or sub-chronic high-dose oral administration of AN resulted in hind limb weakness which was only partially reversible.[45]

Methacrylonitrile (MAN) is used as a replacement for acrylonitrile (AN) in the production of plastics and resins.[46] It is also a component of the smoke from unfiltered cigarettes.[47]

Methacrylonitrile (MAN) is excreted much faster than acrylonitrile (AN)[21] and excreted via the lung to a greater extent.[21] MAN urinary metabolites are more associated with the epoxide.[21] MAN excreted via the lungs is either after epoxide formation or unchanged MAN.[48] Lung tissues also show high levels of MAN at an early time period (5 min).[12] Following oral administration, excretion is rapid up to a dose of 29 mg/Kg after which excretion mechanism(s) become saturated and elimination is greatly slowed.[49]

MAN is metabolized by cytochrome p-450 enzymes to the epoxide intermediate 1-cyano-1-methoxyloxirane.[50] At the same dose in mice, MAN produces more cyanide than acrylonitrile.[51] When MAN was administered to CYP2E1-null mice, there was reduction in cyanide formed[51] while AN administered to the CYP2E1-null mice nearly eliminated the cyanide formation.[51] When a CYP inhibitor not specific to 2E1 was given prior to the MAN administration to the CYP2E1 null mice, this nearly eliminated the cyanide formation.[51] This suggests that cyanide formation in MAN is not specific to CYP2E1 and that other cytochrome p-450 microsomes are involved in the metabolism of MAN.[51]

MAN is known to induce neurotoxicity in rats[52] although sub-chronic oral administration of MAN did not result in observed hind limb paralysis.[45]

Figure 3 - Proposed mechanism of methacrylonitrile (MAN)[48]

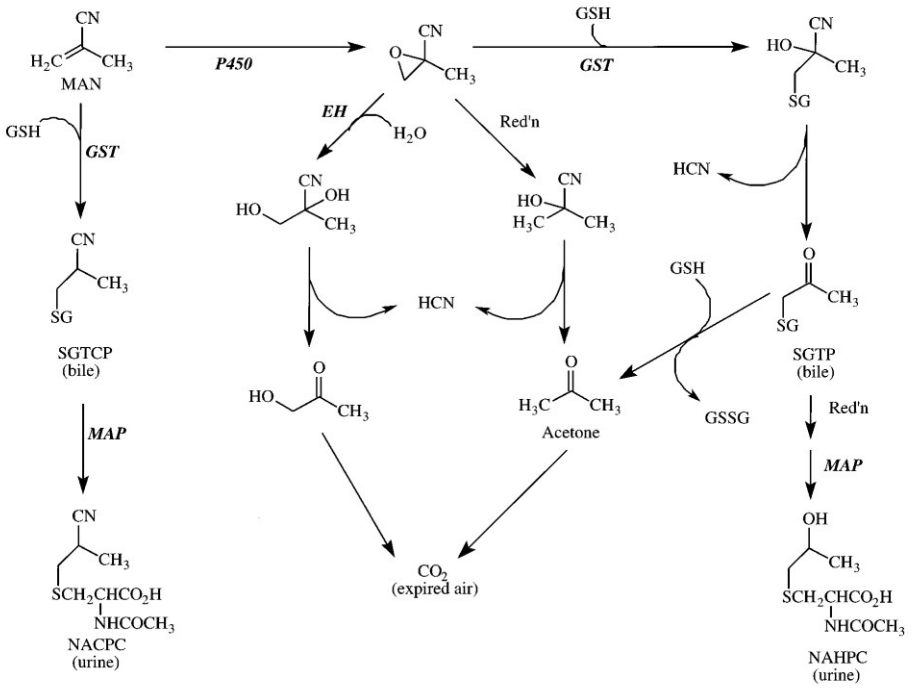
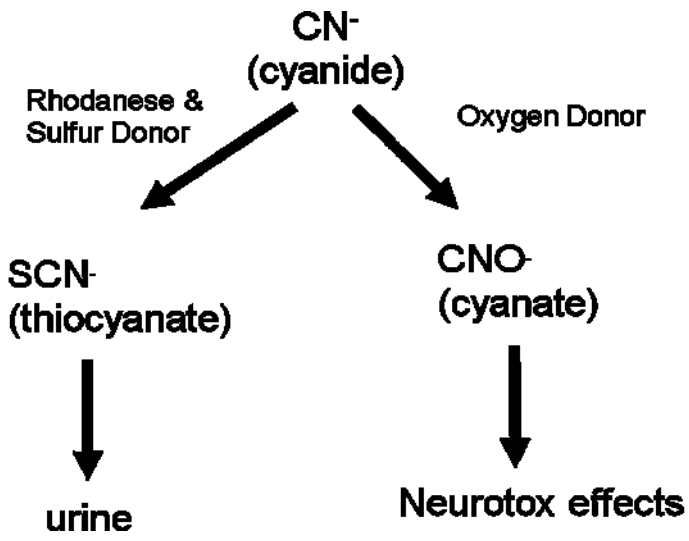


Fig. 1. A metabolism scheme for MAN. Underlined metabolites have been identified. Italicized abbreviations are enzymes or enzyme-catalyzed transformations: EH, epoxide hydrolase; GST, glutathione-S-transferase; MAP, mercapturic acid pathway; P450, cytochrome P-450; Red'n, reduction.

Cyanate

Cyanide (CN) is normally metabolized to thiocyanate (SCN^-) via the sulfur-dependent enzyme rhodanese[53] and then excreted in the urine. When sulfur amino acids are not readily available, cyanide may be converted to cyanate, which is known to cause neurologic effects in humans and animals[53]. **(Figure 4)**

Figure 4. **Metabolism of cyanide**



Sodium cyanate is known to increase hemoglobin's affinity for oxygen and at one point was considered as a treatment for sickle cell anemia.[54] Sodium cyanate acts by carbamylation of the amino terminal valine of hemoglobin. Early oral studies showed no adverse effects in monkeys or dogs to either sodium cyanate or potassium cyanate.[55] People administered sodium cyanate for extended periods as a possible treatment for this disease developed motor and sensory neuropathy.[56] Also, monkeys receiving intramuscular injection of sodium cyanate were found to have permanent hind limb paralysis.[57] **Table 1** indicates there is little difference in the toxicity of sodium cyanate and potassium cyanate and they are often used interchangeably in studies.

Table 1 – Comparison of LD_{50} of sodium and potassium cyanate.

Compound	LD_{50}	Route	Species
Sodium cyanate	260 mg/kg[55]	i.p.	Mouse
Potassium cyanate	320 mg/kg[55]	i.p.	Mouse

Spastic paraparesis is also observed in communities in Africa, presumably due to eating Cassava, a plant that contains cyanide.[58] [59] [60] In all cases, the onset was rapid. The effects were also permanent.[61] High cyanide intake combined with deficient sulfur intake impairs the cyanide to thiocyanate conversion.[59]

Sodium cyanate is able to cross the blood brain barrier. After i.p. administration of radio-labeled sodium cyanate in rats, it was detected in the cerebrospinal fluid (CSF). Within 24 hours, the compound was not found in the CSF suggesting cyanate was metabolized and excreted.[62] Sodium cyanate also has a direct effect on glutathione levels resulting in a 50% decrease in brain GSH concentration within 15 minutes and a 35% decrease in liver GSH levels.[53] Following a single dose of cyanate, the physiological effects range from restlessness at low doses, to hind limb paralysis at medium doses, to noise triggered convulsive seizures at high concentrations.[63] Rats administrated sodium cyanate i.p. for extended periods were also noted to have weakness, particularly of the hind limbs.[64] In humans and rats, the effects of sodium cyanate were not noted for weeks or months after administration. The onset of hind limb paralysis is dose dependent in rats.[65] Even on a diet consisting of low sulfur amino acids (SAA), rats still took two days before hind limb paralysis was observed.[66]

Rats were fed a diet of low sulfur amino acids or a normal diet and then given water that contains potassium cyanide. Over the next four weeks, cyanide, cyanate, and thiocyanate levels were measured. Thiocyanate levels did not change over time or between the groups. With the SAA-free diet, there was only a small change in cyanide levels after 4 weeks. Cyanate levels were consistent with both groups for the first two

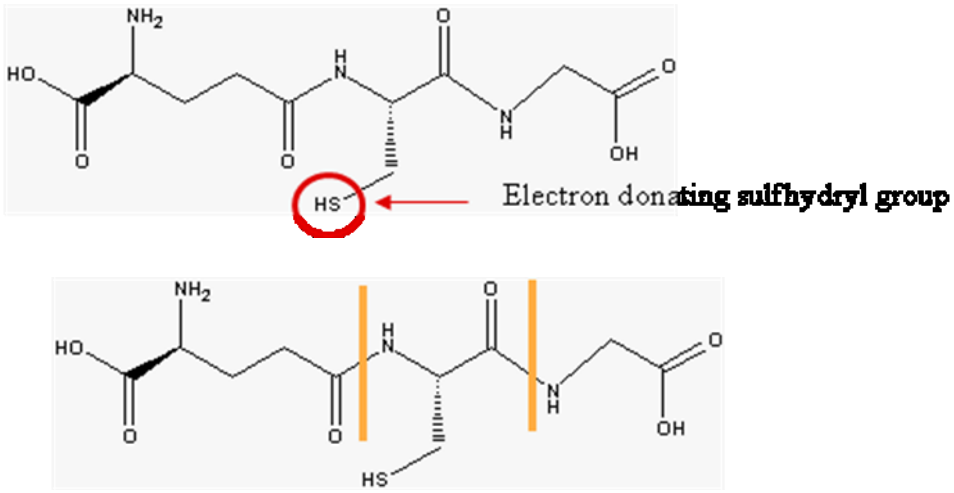
weeks, but the concentration of plasma cyanate in the SAA-free group increased exponentially in weeks three and four. The final concentration of cyanate blood plasma level was 113 nmol/L in the SAA-free diet compared to 29 nmol/L in the regular diet group.[53] No hind-limb effects were noted. The authors' suggested this was due to the study being terminated prior to the limit of cyanide metabolism via thiocyanate being sufficiently saturated.[53] This result suggests that in rats, cyanide can be converted to cyanate. It also shows that with a low sulfur diet, the levels of cyanate were higher.

When rats were fed a cyanate-rich diet for 18 months, all developed motor weakness. The cause was determined to be damage to the myelin sheath, especially the sciatic nerves.[67] More recently, sodium cyanate has been shown to cause an increase in the open probability of large conductance calcium-activated potassium channels (BK_{Ca}) in hippocampal neuron-derived cells.[68] These channels are also referred to as high-conductance calcium-activated potassium (maxi-K) channels. Increasing the opening of these channels would result in a decrease in neuronal excitability.

Glutathione

Reduced glutatione is a critical cellular antioxidant consisting of the tripeptide y-glu-cys-gly (**Figure 5**).[69]

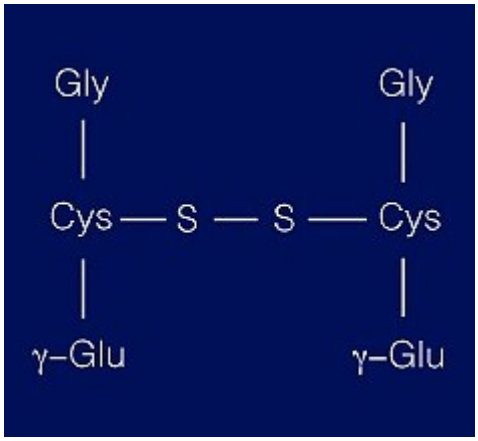
Figure 5. Structure of reduced glutathione (GSH)



y-glu cys gly

Reduced glutathione is typically present in concentrations ranging from 0.1-10 mM and is the most prevalent thiol and the most abundant low molecular weight peptide. In many cells, GSH accounts for over 90% of the total non-protein sulfur.[70] Extensive cell damage or cell death will occur if there is not a sufficient supply of GSH.[71] The first function of GSH is to donate a hydrogen ion to unstable molecules such as reactive oxygen species. As noted in figure 6, two molecules of GSH react to form the oxidized form of glutathione (GSSG).

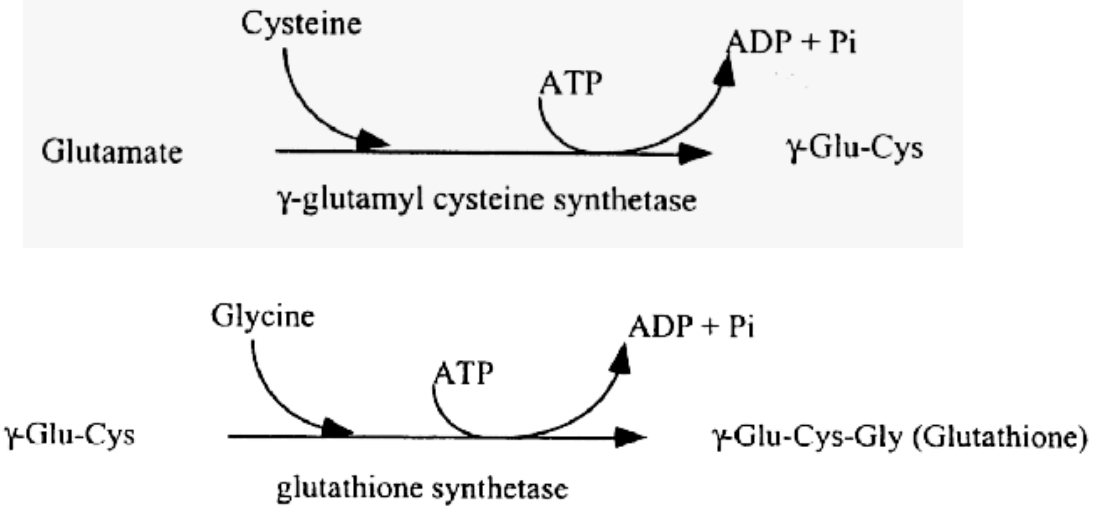
Figure 6. Structure of oxidized glutathione (GSSG)



Another function of GSH is direct conjugation with compounds. During this process, GSH directly binds to the compound and the conjugate is excreted in the urine as mercapturic acid metabolites.

Because GSH is so critical to normal cellular function, there is a substantial ability for the cells to produce GSH. The rate limiting step in the synthesis of GSH is the quantity of available cysteine.[72] **Figure 7** describes the mechanism for the synthesis of GSH

Figure 7 – Synthesis of GSH[73]



Increasing cellular GSH levels is not possible via administration of GSH.[74] This is due to extracellular metabolism and the inability of GSH to cross the cell membrane. A more effective way to increase the amount of cellular GSH is to increase the amount of cysteine available for the cell to produce its own GSH. As an example, it is known that hepatic damage caused by acetaminophen is GSH-dependent.[75] Pre-treatment with a compound known to deplete GSH increased hepatic necrosis in mice.[75] Pretreatment with cysteine, which is known to increase GSH synthesis, results in a reduction in hepatic damage.[75] Only when GSH is severely depleted (greater than 75%), do acetaminophen metabolites covalently bind to other hepatic macromolecules.

Cytochrome P-450

In addition to changes in glutathione levels, it is expected that oxidation may play an important role in the toxic mechanism of 2-CAN. Cytochrome P-450 (CYP) is a superfamily of hemoproteins which are catalysts for the oxidative metabolism of many compounds (endogenous and exogenous) including testosterone, chlorzoxazone, and coumarin.[76] There are dozens of subtypes of CYPs. One way to divide the category of P-450 cytochromes is by family. For example, the CYP2 is associated with drug and steroid metabolism. The family is further divided into subfamilies based on the genes that code each protein. For example, the CYP2E1 protein is encoded by the CYP2E1 gene.

CYP2E1 concentrations are highest in the liver with lower levels in other tissues.[77] CYP2E1 is involved in the metabolism of alcohol to acetaldehyde[78], but has a much lower affinity than alcohol dehydrogenase.[79] The interaction with alcohol is important because alcoholics can have CYP2E1 induced by a factor of 3-5.[80] There is also a large

variation in CYP2E1 activity among people from different ethnic backgrounds, and even specific polymorphisms within ethnic groups.[79] Persons with slower CYP2E1 activity have higher acrylonitrile adduct levels due to less acrylonitrile being oxidized.[81]

In order to limit other potential biological effects of inhibiting multiple CYP subtypes, we needed to select as specific an inhibitor as possible. 5-phenyl-1-pentyne (5P1P) is an inhibitor of CYP2E1 in rat liver.[82] 5P1P is also an inhibitor of CYP2F2, or a mixed inhibitor of CYP2E1 and CYP2F2.[83] [84] [85] The designation of 5P1P as an inhibitor of CYP2F2 is based on the effects of 5P1P which are similar to effects observed in CYP2E2 knockout mice.[86] 5P1P is sometimes administered for weeks, but the least amount of time noted in any study was 1 hour prior to administration of the test compound.[83] Based on a phone discussion with one of the authors (GP Carlson), 100 mg/kg for 1 hour was selected because it would be sufficient for some inhibition without overloading the animal and causing unintended effects.[87]

Several drugs approved for use in humans are known to inhibit cytochrome P-450 including: chlorpromazine, diclofenac, fluphenazine, thioridazine, isoniazid, diallyl sulfide, 4-methyl pyrazole, and disulfiram.[88] Some antifungal azoles, antidepressants, (phenothiazine antipsychotics), benzodiazepines (flurazepam, medazepam) and non-steroidal anti-inflammatory drugs (Diclofenac and Flufenamic acid) are known to have specificity for CYP2E1.[89] In humans, isoniazid has been shown to be an inhibitor of CYP2E1 and, when administered after acetaminophen, reduce the formation of acetaminophen thioether metabolites.[90]

Cyanide

Cyanide is known to be very toxic in humans and animals. Under normal biological metabolism, cytochrome oxidase a3 is needed to transfer electrons from NADH to oxygen in the mitochondria. The cyanide ion inhibits this enzyme by binding to the ferric ion found in the enzyme. This prevents the transport of electrons by cytochrome a3 oxidase to oxygen. This results in the cell no longer being able to aerobically produce ATP. Depending on severity, this effect could cause cell damage, tissue damage, or death.[10] See table 2 for blood concentrations and their effects noted in humans.

Table 2 Blood concentrations of cyanide and associated clinical effects in humans[91]

Cyanide Concentration (µg/mL)	**Signs and symptoms**
0.2 – 0.5	None
0.5 – 1.0	Flushing, tachycardia
1.0 – 2.5	Lack of alertness and orientation
2.5 – 3.0	Coma
>3	Death

Cyanide metabolism is very rapid. In rats, maximum cyanide concentration is observed in 15 minutes while the maximum thiocyanate level is observed in 6 hours.[92] Cyanide is also known to induce rhodanese[93], the main enzyme in the thiocyanate detoxification pathway. (See **Figure 4**) Therefore with low level chronic exposure, the amount of cyanide the body can metabolize is increased.

The traditional treatment for cyanide poisoning is a combination of amyl nitrite, sodium nitrite, and sodium thiosulfate. The amyl nitrite and sodium nitrite generate methemoglobin which then competes with cytochrome c for cyanide. This is the rapid response to cyanide poisoning, but is dangerous because methemoglobin decreases the body's ability to transport oxygen. Sodium thiosulfate converts cyanide to cyanate and is excreted in urine as mercapturic acid metabolites as described previously. The FDA recently approved a new cyanide antidote kit (Cyanokit) which contains hydroxocobalamin, a vitamin B12 precursor. Hydroxocobalamin directly binds to cyanide to form cyanocobalamin (vitamin B12).[94] It does not have the side effects associated with the traditional treatment, but it does interfere with many other drugs and clinical tests.

Materials and Methods

A. Materials and Equipment

1. Facilities

Laboratories – Mellon Hall, Room 405, 419a, and 456

Office – Mellon Hall, Room 419

Animal Care – Animal Care Facility, Bayer Learning Center, Basement

Animal Prep – Animal Care Facility, Bayer Learning Center, Macro surgery Room

2. Animals

For all glutathione, cyanide, and cyanate experiments, male Sprague-Dawley rats were obtained from Hilltop Lab Animals, Inc., Scottdale, Pennsylvania. Initial weight was 200-250g. Animals were given at least one week to acclimate prior to the start of experiment. The animals were housed in a temperature and humidity controlled facility on a 12 hour light/dark cycle. The temperature range was 64-79 ^{o} C and the humidity range between 30-70%. Rats were provided Purina Rat Chow #5001 and water *ad libitum*.

For the hind limb paralysis experiments, male Sprague-Dawley rats originally obtained from Charles River Laboratories International, Inc. were bred at Duquesne and offspring were used. Animal weight at the time of the experiments was 350-550 g. Animals were given at least one week to acclimate prior to the start of experiments. The animals were housed in a temperature and

humidity controlled facility on a 12 hour light/dark cycle. They were provided

Purina Rat Chow #5001 and water *ad libitum*.

3. Chemicals

1-Fluoro-2,4-Dinitrofluorobenzene
Sigma Chemical Co., St. Louis, MO.

2-Chloroacrylonitrile (2-CAN) 99%
Aldrich Chemical Co., Milwaukee, WI.

Acetic Acid, Glacial, ACS reagent, >99.7%
Sigma-Aldrich, St. Louis, MO.

Acetic Acid, Sodium Salt Trihydrate, Anhydrous
Fisher Scientific Co., Pittsburgh, PA.

Ethyl alcohol, absolute, 200 proof, 99.5% ACS reagent
Aldrich Chemical Co., Milwaukee, WI.

Barbituric Acid
Fluka - Sigma Aldrich, St. Louis, MO.

Bathophenathroline Disulfonic Acid (DL-Buthionine-[S,R]-sulfoximine)
Sigma Chemical Co., St. Louis, MO

Chloramine-T
Riedel-de Haen, Honeywell

γ-glutamyl glutamate
Sigma Chemical Co., St. Louis, MO.

γ-glutamyl glutamate
MP Biomedicals, LLC, Solon OH

BD Vacutainer Sodium Heparin (NH) 143 USP Units plus blood collection tubes
BD., Franklin Lakes, NJ

M-Cresol Purple Indicator Grade 99.8%
Sigma Chemical Co., St. Louis, MO.

Methanol, CHROMASOLV plus, for HPLC, > 99.9%
Sigma-Aldrich, St. Louis, MO.

Perchloric Acid, 70%, ACS reagent
Sigma-Aldrich, St. Louis, MO.

Potassium Hydroxide Reagent ACS (Pellets)
Fisher Scientific Co., Pittsburgh, PA.

Potassium Hydrogen Carbonate
Fisher Scientific Co., Pittsburgh, PA.

Sodium Chloride 0.9%
Baxter Medical Supplies, Deerfield, IL.

Sodium Hydroxide Pellets
Sigma-Aldrich, St. Louis, MO.

10M Sodium hydroxide solution

Sodium Phosphate Monobasic Dihydrate
Fisher Scientific Co., Pittsburgh, PA.

Ethyl acetate, 99.5+%, ACS reagent
Sigma-Aldrich, St. Louis, MO.

Hydrochloric Acid, Certified ACS
Fisher Scientific Co., Pittsburgh, PA.

Sulfuric Acid, 95-98%, ACS reagent
Sigma-Aldrich, St. Louis, MO.

Water, HPLC Grade
Sigma Chemical Co., St. Louis, MO.

Potassium Cyanide
Sigma-Aldrich, St. Louis, MO.

Pyridine, 99+%, ACS reagent
Sigma-Aldrich, St. Louis, MO.

Ammonium Hydroxide, certified ACS Plus
Fisher Scientific Co., Pittsburgh, PA.

Iodoacetic Acid, sodium salt >98%
Sigma Chemical Co., St. Louis, MO.

Glutathione, Reduced, 98%
Sigma Chemical Co., St. Louis, MO.

Glutathione, Oxidized, 98%
Sigma Chemical Co., St. Louis, MO.

Potassium phosphate, Dibasic, Anhydrous
Sigma Chemical Co., St. Louis, MO.

Sodium Phosphate, Dibasic, Anhydrous Enzyme Grade
Fisher Scientific, Pittsburgh, PA

Benzoyleneurea, 97%
Aldrich, St. Louis, MO.

5-phenyl-1-pentyne, 98+%
Lancaster Synthesis, Inc., Pelham NH

Isoflurane (Aerrane)
Baxter Healthcare, Deerfield, IL

Potassium cyanate
Fluka - Sigma Aldrich, St. Louis, MO.

Anthranilic Acid
Fluka - Sigma Aldrich, St. Louis, MO.

Liquid Nitrogen
Airgas Inc., Radnor, PA.

Sodium Acetate, trihydrate
Acros, Thermo Fisher Scientific, Rochester, NY

4. Materials

20 mL Cryogenic specimen containers (Nalgene)
Fisher Scientific Co., Pittsburgh, PA.

Mortar and pestle
Duquesne University, Pittsburgh, PA.

1.5 mL Microcentrifuge tubes
Fisher Scientific Co., Pittsburgh, PA.

2.0 mL Microcentrifuge tubes
Fisher Scientific Co., Pittsburgh, PA.

20 µl, 100 µl, 200 µl and 1000 µl Micropipettes
Rainin Instrument Co., Woburn, MA.

200µLand 1000µLMicropipette tips
Rainin Instrument Co., Woburn, MA.

Conway microdiffusion dishes
Duquesne University, Pittsburgh, PA.

5 mL Volumetric flasks
Fisher Scientific Co., Pittsburgh, PA.

Falcon Blue Max Jr. 15mL polypropylene conical tube (17x20 mm)
Becton Dickinson Labware, Franklin Lakes, NJ

Fisherbrand Disposable Culture Tubes, Borosilicare Glass (12x75 mm)
Fisher Scientific Co., Pittsburgh, PA.

Varian Bond Elut Solid Phase Extraction Mega BE-PRS, 1GM, 6ML
Varian Inc., Santa Clara, CA

Waters Sep-Pak Vac 6cc (500 mg) C18 Cartridges
Waters Corporation, Milford, MA.

Minisart 0.45um single use syringe filter, hydrophilic
Vivascience, Hannover Germany

Pierce screw Cap Septum Vials
Pierce Biotechnology, Rockford IL

Durapore membrane filters, 0.45um HV
Millipore, Billerica, MA

10cc syringe with luer-lok tip
Becton Dickinson, Franklin Lakes, NJ

Kimble Glass tubes, culture, S/T, R.B. 16x25mm
Thermo Fisher Scientific, Rochester, NY

Fisher Brand Glass, serological pipets, 10mL
Fisher Scientific Co., Pittsburgh, PA.

Millipore polypropylene filters (type HV, pore size 0.45 μm)
Fisher Scientific Co., Pittsburgh, PA.

5. Equipment

HPLC column: 20-cm × 4.6-mm-i.d. column with 5-μm Exsil silica derivatized with 3-aminopropyltriethoxy silane
CEL Associates, Houston, TX.

HPLC column for glutathione: 3-aminopropyl, 5um
Custom LC, Inc., Houston TX

HPLC column for cyanate: Kromasil 100-5C18 25cm x 4.6mm (i.d.)
HiChrom Limited, Reading England

HPLC prefilter for cyanate: Kromasil
HiChrom Limited, Reading England

Waters 510 HPLC Pump
Waters Corporation, Milford, MA.

Waters 501 HPLC Pump
Waters Corporation, Milford, MA.

Waters Automated Gradient Controller
Waters Corporation, Milford, MA.

Perkin Elmer 785A UV/Vis detector
Perkin Elmer, Norwalk, CT.

Perkin Elmer Series 200 Fluorescence detector
Perkin Elmer, Norwalk, CT.

Perkin Elmer Series 200 Autosampler
Perkin Elmer, Norwalk, CT.

Perkin Elmer Nelson Network Chromatography Interface
Perkin Elmer, Norwalk, CT.

Dell OptiPlex GX1
Dell Corporation, Austin, TX

1L Vacuum filtration flask
Fisher Scientific Co., Pittsburgh, PA.

Millipore filtration apparatus (to vacuum flask)
Fisher Scientific Co., Pittsburgh, PA.

Pierce Reacti-Therm III heating module
Pierce Corporation, Rockford, IL

Pierce Reacti-Vap II
Pierce Corporation, Rockford, IL

Varian Cerex SPE processor

Corning pH meter 220
Corning Corporation, Corning, NY

Fisher Scientific Marathon 16KM centrifuge
Fisher Scientific Co., Pittsburgh, PA.

Fisher Scientific Centric Centrifuge
Fisher Scientific Co., Pittsburgh, PA.

Reliable Scientific shaker
Reliable Scientific Inc, Nesbit, MS

Fisher Scientific ACCU-124 balance
Fisher Scientific Co., Pittsburgh, PA.

Beckman DU-530 Life Science UV/Vis Spectrophotometer
Beckman Coulter, Danvers, MA

Precision Reciprocal Shaking Bath
Thermo Fisher Scientific, Rochester, NY

Fischer Scientific Sonic Dismembrator model 100
Fisher Scientific Co., Pittsburgh, PA.

Isoflurane vaporizer
A.M. Bickford, Wales Center, NY

6. Computer Software

Microsoft Word
Microsoft Corporation, Redmond, WA

Microsoft Excel
Microsoft Corporation, Redmond, WA

Microsoft Powerpoint
Microsoft Corporation, Redmond, WA

Turbochrom Version 6.3.2
Perkin Elmer, Waltham, Massachusetts

GraphPad Prism 5 for Windows
GraphPad Software, La Jolla, California

Chem Draw 3D Pro 12.0
CambridgeSoft, Cambridge, Massachusetts

B. Methodology and Procedures

1. Hypothesis

Hind limb paralysis resulting from acute 2-CAN exposure is the result of cyanate formation. Blockade of 2-CAN metabolism by cytochrome P-450 enzyme inhibition utilizing 5P1P will attenuate 2-CAN toxicity.

2. Specific Aims

a. Determine whether cyanate is formed in rat tissues following 2-CAN administration.

b. Determine whether the cytochrome P-450 inhibitor 5P1P administered prior to 2-CAN, will reduce cyanate levels.

c. Determine whether reduced cyanate levels are associated with a delay in the onset of hind-limb paralysis.

3. Experimental Design

To determine the effects of CYP inhibition, the following experimental design was used. All experiments using animals were conducted with standards consistent with the Animal Welfare Act and approved by the Institutional Animal Care and Use Committee of Duquesne University. There were 4 groups of male Sprague-Dawley rats with approximately 10 animals per group.

Figure 8 – Experimental Design

4. Sample collection

a. Tissue glutathione, blood cyanide, and plasma cyanate collection

As described in **Figure 8**, male Sprague-Dawley rats were divided into four groups. The first group received vehicle (de-ionized water) at a dose of 1 mL per 100 g animal weight. The second group received 40 mg/Kg 2-CAN. The third group received 100 mg/kg of 5-phenyl-1-pentyne (5P1P). The fourth group received 100 mg/kg 5P1P followed one hour later with 40 mg/kg 2-CAN. Dosages were administered i.p. between the hours of 8 and 10 AM. This time range was selected because of the peak time of liver glutathione (GSH) diurnal rhythm.[95] After 3 hours, the animals were anesthetized via 3%

isofluorane. While under anesthesia, the abdomen was opened with scissors and the upper left lobe of the liver and the left lung were removed. The tissues were rinsed with saline, cut into smaller pieces, and immediately placed in liquid nitrogen. Blood was removed via cardiac puncture, placed in heparinized vials, and then placed on ice. Cardiac puncture was used to euthanize the animals. One mL of whole blood was used for cyanide analysis and the remainder of the whole blood was centrifuged at 2,500 rpm for 20 minutes (Calculated G-force of 908 xG). The supernatant (plasma) was immediately used for cyanate analysis.

Based on the research of Mostowy[96], 40 mg/kg 2-CAN was selected because of observed hind-limb paralysis and glutathione effects. Liver and lung tissues were selected because glutathione levels were more sensitive to exposure to 2-CAN than heart and kidney.[96] Previous studies showed that three hours was the time of maximum GSH depletion in the liver and nearly maximum GSH depletion in lung.[96]

5-phenyl-1-pentyne was selected as the cytochrome P-450 inhibitor because it inhibits both CYP2E1 and CYP2F2. CYP2F2 is associated with metabolism in the lungs and MAN is excreted in the lungs at a higher rate than AN. 5P1P was also effective in reducing toxicity in styrene metabolism which is also dependent on lung metabolism.

5. Tissue Glutathione, Blood Cyanide, and Plasma Cyanate Analysis

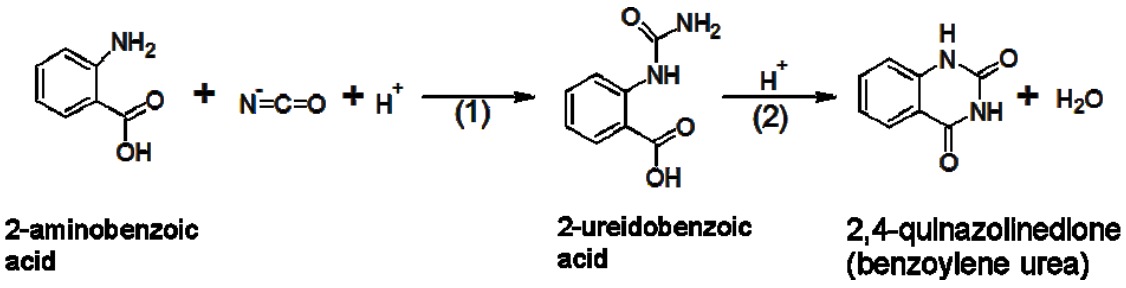
a. **Plasma Cyanate analysis**.

Plasma cyanate was determined using high performance liquid chromatography (HPLC) according to the method of Lundquist.[97] The method involves the derivatization of cyanate with 2-aminobenzoic acid (**figure 9**) to form the stable fluorescent compound 2,4(1H,3H)-Quinazolinedione. One mL of the plasma supernatant was placed into a

glass test tube. To the tube, 1 mL of 0.2 mL/l sodium phosphate buffer (pH 7.4) was added. The pH of the solution was then adjusted to 4.7 by adding approximately 60 µL of 4 mol/L acetic acid. 2.1 mL of 40 mmol/l of 2-aminobenzoic acid (adjusted to pH 4.7 with sodium acetate) was added and the entire solution mixed. The tube was then incubated in 40°C water for 10 minutes. The reaction was stopped by placing the tube in an ice bath. A three mL aliquot was then placed into a new glass tube containing 3 mL of 6 mol/L sulfuric acid. This tube was then placed in a boiling water bath for 1 minute and 15 seconds. Again, the reaction was stopped by placing the tube into an ice bath. The tube was then spun on a centrifuge at 2,000 rpm for 20 minutes. Using a 10 mL syringe, the supernatant was then passed through a Minisart® filter and placed into new tubes. Four mL was added to a new tube containing three mL of ethyl acetate. The tube was capped and mixed for five minutes. The tube was centrifuged for 10 min at 2,000 rpm. The organic phase was extracted and placed into a new glass test tube and three mL of ethyl acetate was added. This process was repeated two more times, each time combining the organic phase into another tube. The organic phase (which contained the sample) was then dried on a 40^{o} C heating block with dry nitrogen gas passing over the tubes. The residue was then dissolved by adding a mixture of 2 mL of water and 60 µL of 5 mol/l HCl. A 6 mL Mega Bond® Elut column was then prepared by wetting with 3 mL of water. The sample was then added to the column and pushed through with nitrogen. The effluent was collected. The column was then washed twice with 2 mL of water with the effluent being collected each time. To the combined effluent, 0.5 mL of 0.1 mol/L sodium phosphate buffer (pH 7.4) was added. The pH was then adjusted to 6-8 by adding a few drops of three mol/L NaOH. A Sep-Pak® C-18 cartridge was then

prepared by washing with 3 mL of MeOH and then with three mL of water. The sample was then added to the column and pushed through with nitrogen. The column was then washed with three mL of water. The effluent was not collected due to the fact that the derivatized sample was still on the column. To extract the sample from the column, three mL of MeOH was added and pushed through with nitrogen. The MeOH effluent was collected and dried. The sample was then dissolved in 0.5 mL of mobile phase and analyzed by HPLC with fluorescence detection (excitation of 313nm and emission of 353). The quinazolinedione derivative was determined on the HPLC with a runtime of less than 30 minutes. The quantification of cyanate in plasma was calculated based on a standard curve. For standards, it was not necessary to centrifuge and filter prior to extraction with ethyl acetate.

Figure 9 - Derivatization of cyanate for HPLC analysis



See **Appendix A** for a description of the solutions used in cyanate analysis

See **Appendix B** for the method to prepare the standard curve.

See **Appendix C** for the method to analyze on a HPLC

See **Appendix D** for the calculation to determine the cyanate concentration

b. Tissue Glutathione Analysis.

The glutathione level of each tissue was determined using the method of Farris and Reed High Performance Liquid Chromatography (HPLC).[98] The tissue was removed from the liquid nitrogen, placed in the mortar and pestle that were cooled with liquid nitrogen. The tissue was quickly ground, returned to the cryogenic container, and placed back into the liquid nitrogen. This was done for each liver and lung sample.

Nine hundred μL of the PCA/BPDS working solution was added to a 2 mL microcentrifuge tube. The purpose of the PCA was to keep the GSH in its reduced form. BPDS acted as a metal chelator. One hundred mL of the γ-Glu-Glu working solution was added as an internal standard. The purpose of using the γ-glutamyl glutamate as the internal standard for both of the samples is to increase the accuracy of the method to greater than 95% for both the GSH and GSSG determinations. The microcentrifuge tube containing the solution was then placed on a balance and net to zero. Approximately 80-100 mg of ground tissue was removed and placed in the tube. The excess was wiped from the edges and the weight noted. The tube was then sonicated on ice for 10 seconds and returned to the liquid nitrogen. The samples were then allowed to thaw in refrigerator. This took several hours. The samples were each centrifuged at 13,000 X for 10 minutes and 200 μL of the supernatant was removed and placed into a new 1.5 mL microcentrifuge tube.

To each of the supernatant samples, 50 μL of the IAA/m-cresol purple solution was added. The samples were then adjusted to have a pH of 8-9 by using $KOH/KHCO_3$ Solution. The $KOH/KHCO_3$ solution was added at no more than 50 μL increments to prevent the sample from bubbling over. A total of 130 μL was added to each sample.

The samples were then covered in a thick black cloth and put into a laboratory drawer for one hour. The purpose of this step was for the iodoacetic acid to react with the thiols in order to form S-carboxymethyl derivatives. The purpose of the m-cresol purple was not only to function as a pH indicator, but also as a preservative.

The reaction of the iodoacetic acid with the thiols and forming S-carboxymethyl derivatives of thiols eliminated the potential for thiol disulfide interchange. An equal volume of FDNB solution was added to each sample (380 µL). Samples were then incubated in a dark environment at room temperature for 18-24 hours. The tubes were covered by a thick black cloth and put into a lab drawer in order to keep the samples in complete darkness. After 24 hours in room temperature darkness, the samples were placed in a 13,000 X centrifuge for 20 minutes. One hundred µL of the supernatant was added to the autosampler tubes and placed into the autosampler. In addition to samples, a standard curve, GSH only, and GSSG only samples were analyzed via HPLC with UV/Vis detection at a wavelength of 365nm. Each run took about 45 minutes. Using the standard curve, the concentrations of GSH and GSSG were calculated for each sample.

Appendix E is a description of the working solutions used during the glutathione analysis.

Appendix F is a description of the method used to prepare GSH and GSSG standards.

Appendix G is a description of solvent and gradient system used during the glutathione analysis.

Appendix H is a description of the calculations used to determine the levels of GSH and GSSG.

Appendix I is a description of column and HPLC retention times.

Appendix J is representative HPLC chromatographs and standard curves.

c. **Blood Cyanide Analysis.**

Whole Blood – Following anesthesia with Isoflurane, a 10 mL syringe with a 20 G needle was used to extract whole blood from the heart of the animal. For most animals, this quantity was 7-9 mL. The whole blood was then added to a heparinized vial and placed on ice. The whole blood was then analyzed for cyanide by a method similar to that of Feldstein and Klendshoj.[99]

The method utilizes a procedure of microdiffusion in which cyanide is liberated as hydrogen cyanide from blood. A Conway microdiffusion dish was used for this method. This is a flat circular dish with two circular walls dividing the dish into three wells referenced as the outer well, the middle, and the center well. The inner well is slightly shorter than the outer well to allow chemicals to be separated. There is a lid that fits into the outer well. It is used to prevent the release of gases.

The general principle of the analysis is mixing blood with sulfuric acid to release hydrogen cyanide gas (HCN). The cyanide gas will then be absorbed by the sodium hydroxide in the center well. Diffusion typically takes 2-4 hours at room temperature. The HCN will then be converted to cyanogens chloride by treating the sodium hydroxide/cyanide solution with chloramine T and reacting the cyanogens chloride with pyridine and barbituric acid (2,4,6-trioxohexahydropyremidine) resulting in the formation of a blue dye. The dye is then quantitated by spectrophotometry. The specific procedure to this method is as follows:

To seal the lid onto the Conway diffusion dish, 1 mL of 0.2 N H_2SO_4 was added to the outer well. One mL of whole blood containing heparin was added to one side of the middle well of the dish. 0.5 mL of 10% H_2SO_4 was added to the other side of the middle well, ensuring that reagents did not come into contact with one another. 0.2 mL of 0.1 NaOH was added to the center well. The lid was then applied, rotated and lifted slightly to confirm a tight seal. The plate was then swirled slightly to ensure the sulfuric acid solution mixed well with the heparinized blood, and allowed to diffuse at room temperature for 3 hours.

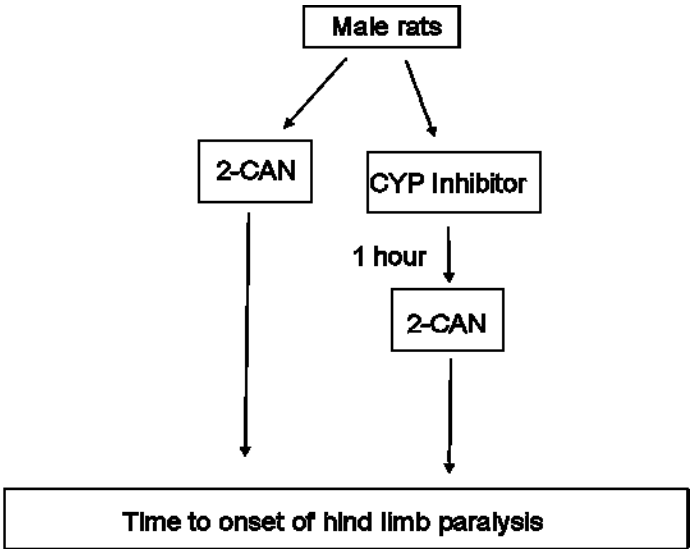
Once diffusion was concluded, the contents of the center well were transferred to a 5 mL volumetric flask. The center well was rinsed twice with 0.25 mL of sodium hydrogen phosphate reagent and added both times to the same volumetric flask. A volume of 0.25 mL of chloramine T reagent was then added to the flask and mixed. This solution was allowed to sit for three minutes. A volume of 0.1 mL of barbituric acid reagent was added to the flask, mixed, and allowed to stand for five minutes. Deionized water was then added to the flask to bring the total volume to five mL. The absorbance was then measured using an ultraviolet spectrophotometer at a wavelength of 580 nm. The concentrations of the standards were 0.01 µg/mL, 0.1 µg/mL, 0.2 µg/mL, and 0.5 µg/mL. A blank was also run.

See **Appendix K** for description of the working solutions used in the blood cyanide analysis.

6. Measurement of hind-limb paralysis

Male Sprague-Dawley rats were randomly divided into 2 groups and weighed (350-550g). The first group (n=5) was dosed via i.p. injections with 40 mg/kg of 2-CAN. The second group (n=5) was administered 100 mg/kg of 5P1P, i.p. 1 hour prior to receiving 40 mg/kg of 2-CAN via i.p injection. Immediately after dosing with 2-CAN, the animal was returned to the plastic cage and a timer was started. The animal was then observed for the first onset of hind-limb paralysis. Normally a rat would move its front limbs in combination with its hind limbs. With 2-CAN induced hind limb paralysis, the animal will move its front limbs but not immediately move the rear. The animal then experiences an unnatural extension of the hind legs. This was noted as the time of onset of hind-limb paralysis. There were also treatment groups for vehicle and 5P1P alone for a physiological baseline.

Figure 10 – Experimental Design for hind-limb paralysis



7. Statistical Analysis

Values are expressed as mean ± S.E.M.. For glutathione, cyanide, and cyanate analysis, a one-way analysis of variation (ANOVA) was performed followed by a Newman-Keuls' multiple comparison test *post-hoc*. For the onset of hind-limb paralysis, a t-test was utilized. For all results, a "*p*-value" of less than 0.05 was considered significant. GraphPad Prism 5.03 (GraphPad Software Inc.) was the software program used.

III. Results and Discussion

A. Results

To test the hypothesis that 2-CAN toxicity is associated with cyanate formation and whether a CYP inhibitor (5P1P) would provide a protective mechanism, male Sprague-Dawley rats were injected i.p. with vehicle (deionized water), 2-CAN (40 mg/kg), 5P1P, or 5P1P followed by 2-CAN as described in the Methodology and Procedures section. In a second experiment, male Sprague-Dawley rats were injected i.p. with 2-CAN (40 mg/kg) or 5P1P (100 mg/kg) followed by 2-CAN as described.

1. Gross animal observations

In the control and 5P1P treated animals, there were no unusual observations. In 2-CAN treated animals, immediately following injection they displayed piloerection. Within 5 minutes there was reduced movement, eye closing, and shallow breathing. Hind limb paralysis was noted around this same time. When the abdomen was opened to remove the liver and lungs, there was an excessive amount of fluid in the peritoneal cavity. Lungs were a coral color and the stomach and intestines were enlarged. The group of animals that received 5P1P prior to 2-CAN had many of the same gross effects as 2-CAN, but was observed to be at a lesser extent. These animals had some reduction in movement, some eye closing, and less pronounced shallow breathing. Hind limb paralysis was also observed but the effects were not as dramatic. There was some fluid in the peritoneal cavity. Lungs were a coral color and the stomach and intestines were slightly enlarged.

2. Tissue GSH levels.

Previous work from Mostowy showed that 40 mg/kg of 2-CAN (i.p.) will deplete lung glutathione level by 31% after 3 hours. In addition to repeating the work by Mostowy, this study will determine the effect of a CYP inhibitor (5P1P) administered 1 hour prior to 2-CAN. It is expected the animals administered 5P1P prior to 2-CAN would have more GSH depletion compared to 2-CAN alone because the toxic metabolic pathway would shift from oxidation (via CYP) to conjugation with GSH. **Figure 11** shows the effects of 2-CAN on GSH levels in lung with and without 5P1P. There was no change in the vehicle compared to the 5P1P only group. The decrease in GSH in the 2-CAN group and the 5P1P + 2-CAN group were both statistically significant compared to vehicle. For the 2-CAN group, the lung GSH concentrations decreased from 1866 µmol/g tissue to 1480 µmol/g tissue; a decrease of about 20%. For the 5P1P + 2-CAN group the lung GSH concentration decreased from 1866 µmol/g tissue to 1432 µmol/g tissue. This is a decrease of approximately 23%. There was no significant different between the GSH concentrations in the 2-CAN group and the CYP + 2-CAN group.

Previous work from Mostowy showed little increase in lung GSSG levels after 40 mg/kg 2-CAN administration (i.p.) It would be expected that 5P1P administered prior to 2-CAN would not significantly increase GSSG concentrations because on the proposed toxic mechanism, very little GSH is oxidized to GSSG and inhibition of CYP would not be expected to change this. **Figure 12** shows the effect of 2-CAN on the GSSG levels in the lung with and without 5P1P. There was not a statistically significant increase in

GSSG concentrations in the 5P1P and 5P1P + 2-CAN groups compared to vehicle and 2-CAN.The concentration of GSH in the rat lung was many times greater than the concentration of GSSG. To illustrate this, **Figure 13** shows the relative quantity of lung GSH compared to GSSG in the different groups.

Figure 11. The effect of 2-CAN and 5P1P on lung GSH levels

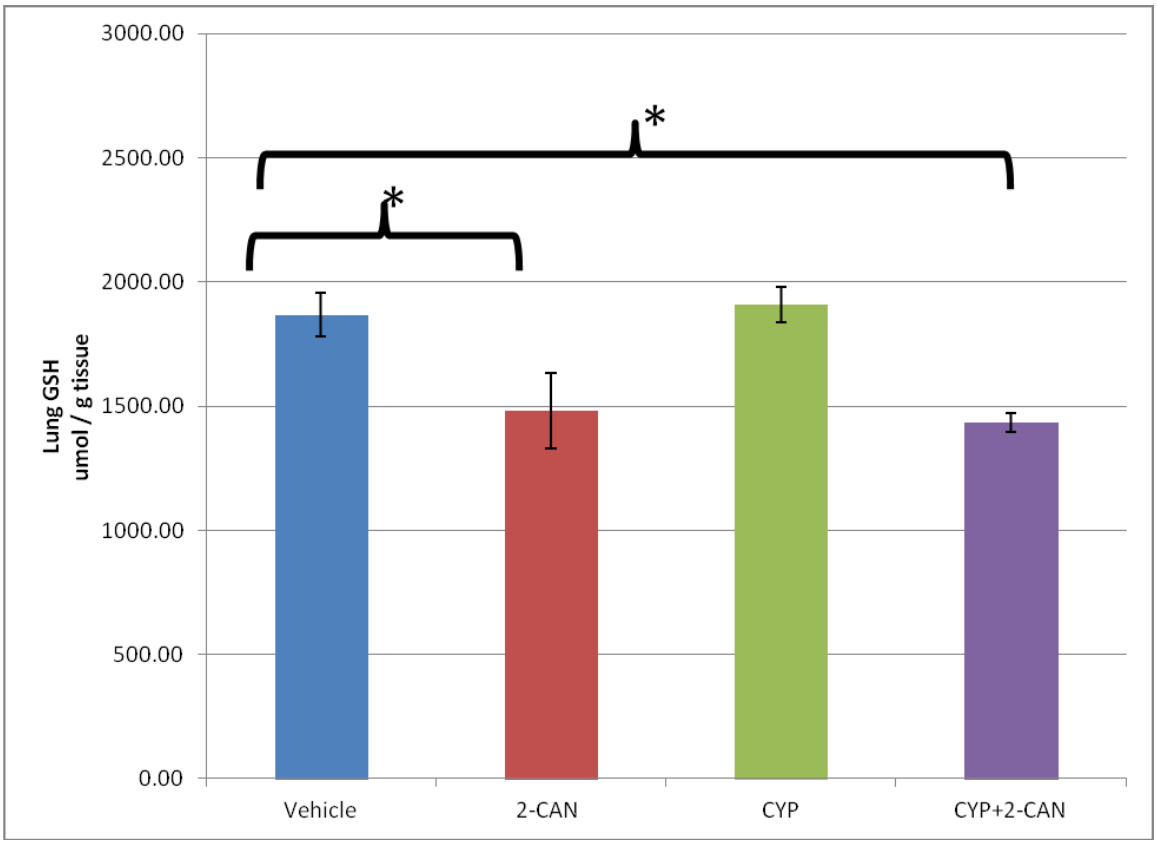


Figure 11. Comparison of GSH levels in the lungs of rats administered vehicle (i.p.); 2-CAN (40 mg/kg i.p); a CYP inhibitor (5P1P); or a CYP inhibitor (5P1P) 1 hour prior to 2-CAN (40 mg/kg i.p). Tissue was removed 3 hours after the last injection. Each bar graph represents the mean ± SEM of 9-12 animals performed in duplicate. Data were analyzed by 1-way ANOVA followed by a Newman-Keuls' post-hoc test where (*) significance indicates $p<0.05$

Figure 12. The effect of 2-CAN and 5P1P on lung GSSG levels

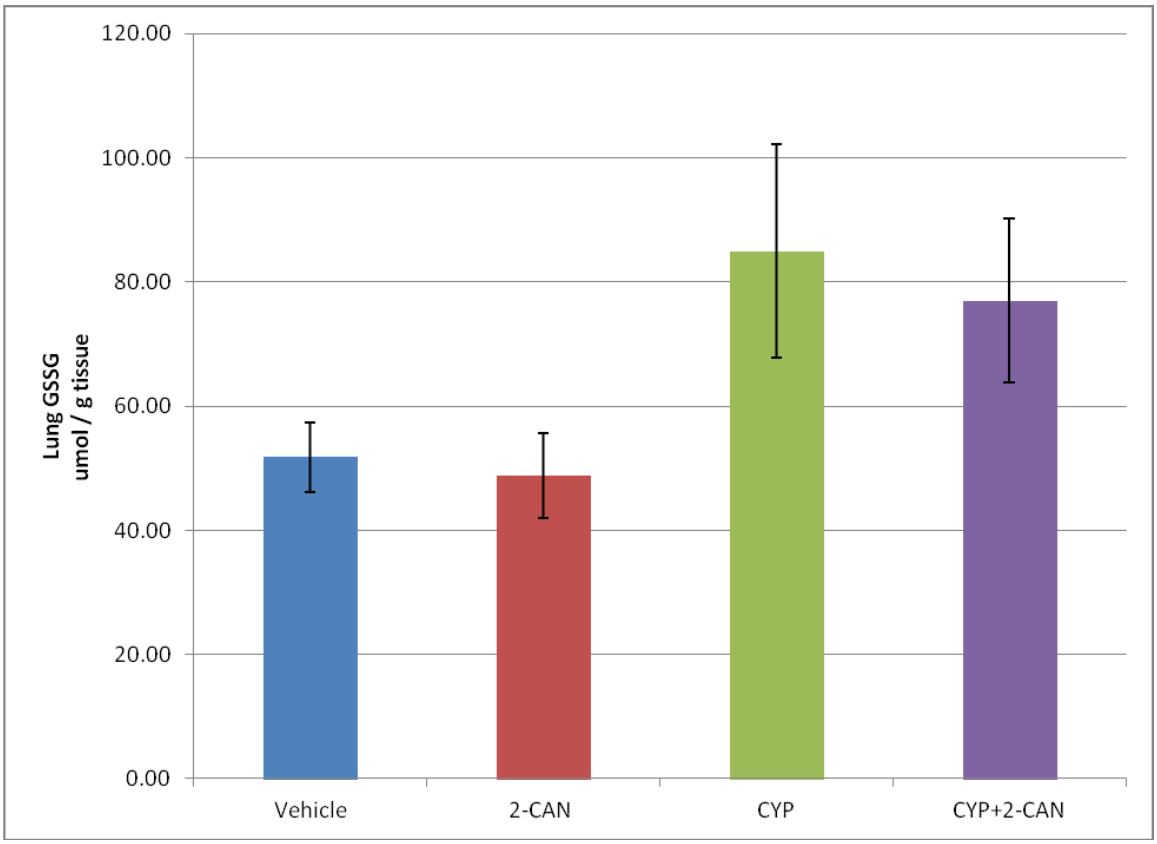


Figure 12. Comparison of GSSG levels in the lungs of rats administered vehicle (i.p.); 2-CAN (40 mg/kg i.p); a CYP inhibitor (5P1P); or a CYP inhibitor (5P1P) 1 hour prior to 2-CAN (40 mg/kg i.p). Tissue was removed 3 hours after the last injection. Each bar graph represents the mean ± SEM of 7-11 animals performed in duplicate. Data were analyzed by 1-way ANOVA followed by a Newman-Keuls' post-hoc test. There was no statically significant change between any of the groups.

Figure 13. Relative amounts of GSH and GSSG levels in the lung following 2-CAN and 5P1P administration

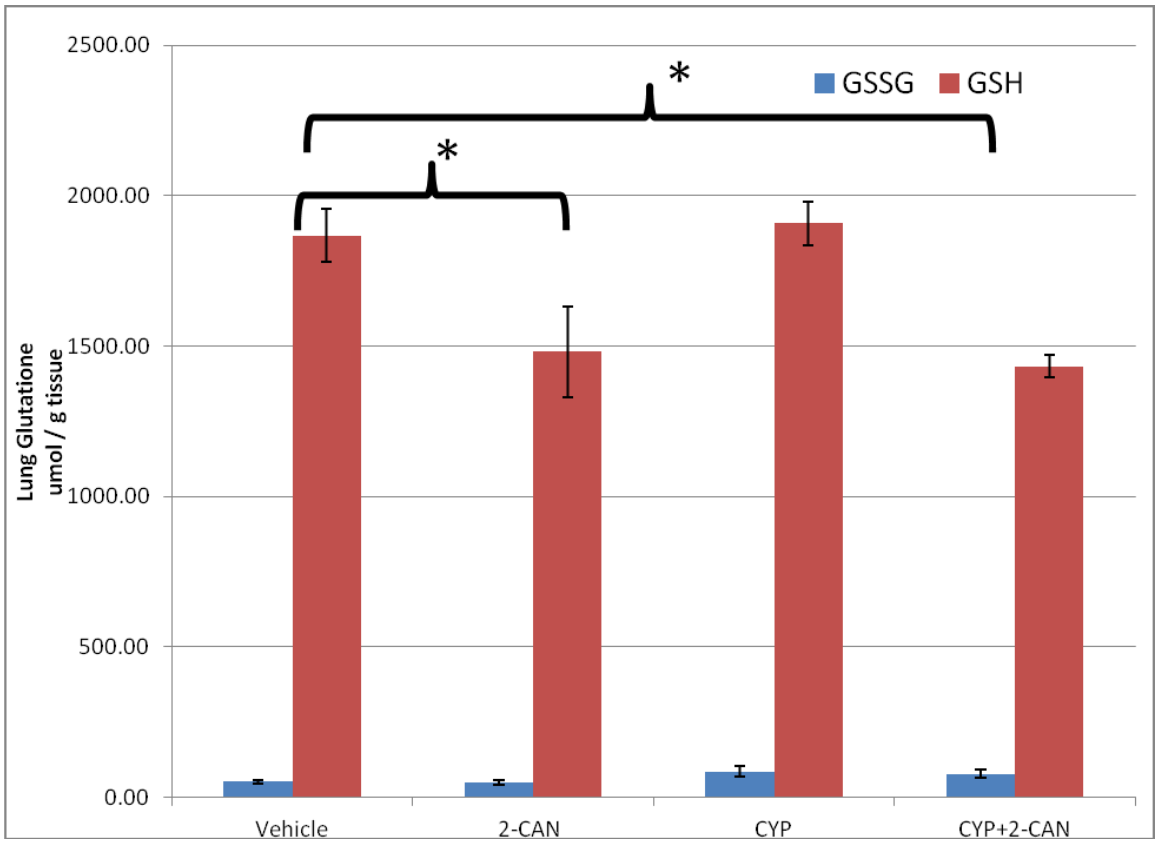


Figure 13. Relative amounts of GSH and GSSG in the lungs of rats administered vehicle (i.p.); 2-CAN (40 mg/kg i.p); a CYP inhibitor (5P1P); or a CYP inhibitor (5P1P) 1 hour prior to 2-CAN (40 mg/kg i.p). Tissue was removed 3 hours after the last injection. Each bar graph represents the mean ± SEM of 7-12 animals performed in duplicate. Data were analyzed by 1-way ANOVA followed by a Newman-Keuls' post-hoc test where significance (*) indicates $p<0.05$

Previous work from Mostowy showed that 40 mg/kg of 2-CAN (i.p.) will deplete liver glutathione level by 42% after 3 hours. In addition to repeating the work by Mostowy, this study will determine the effect of a CYP inhibitor (5P1P) administered 1 hour prior to 2-CAN. It is expected the animals administered 5P1P prior to 2-CAN would have more GSH depletion compared to 2-CAN alone because the toxic metabolic pathway would shift from oxidation (via CYP) to conjugation with GSH. **Figure 14** shows the effect of 2-CAN on the GSH levels in the liver with and without 5P1P. There was no change in vehicle control group compared to the 5P1P only group. The decrease in the 2-CAN group and the 5P1P + 2-CAN group were both statistically significant compared to vehicle. For the 2-CAN group, the liver GSH concentration decreased from 6989 μmol/g tissue to 3913 μmol/g tissue. This is a decrease of approximately 44%. For the 5P1P + 2-CAN group the lung GSH concentration decreased from 6989 μmol/g tissue to 3524 μmol/g tissue. This is a decrease of approximately 50%. There was no significant different between the GSH concentration in the 2-CAN group and the 5P1P + 2-CAN group.

Previous work from Mostowy showed little increase in liver GSSG levels after 40 mg/kg 2-CAN administration (i.p.) It would be expected that 5P1P administered prior to 2-CAN would not significantly increase GSSG concentrations because on the proposed toxic mechanism, very little GSH is oxidized to GSSG and inhibition of CYP would not be expected to change this. **Figure 15** shows the effect of 2-CAN on the GSSG levels in the liver with and without 5P1P CYP inhibitor. Although there appears to be an increase in GSSG concentrations in the 2-CAN group compared to vehicle, CYP, and CYP + 2-CAN, the increase was not statistically significant.

The concentration of GSH in the rat lung was many times greater than the concentration of GSSG. To illustrate this, **Figure 16** shows the relative quantity of liver GSH compared to GSSG in the different groups. The quantity of GSSG compared to GSH is vastly different.

Figure 14. The effect of 2-CAN and 5P1P on liver GSH levels

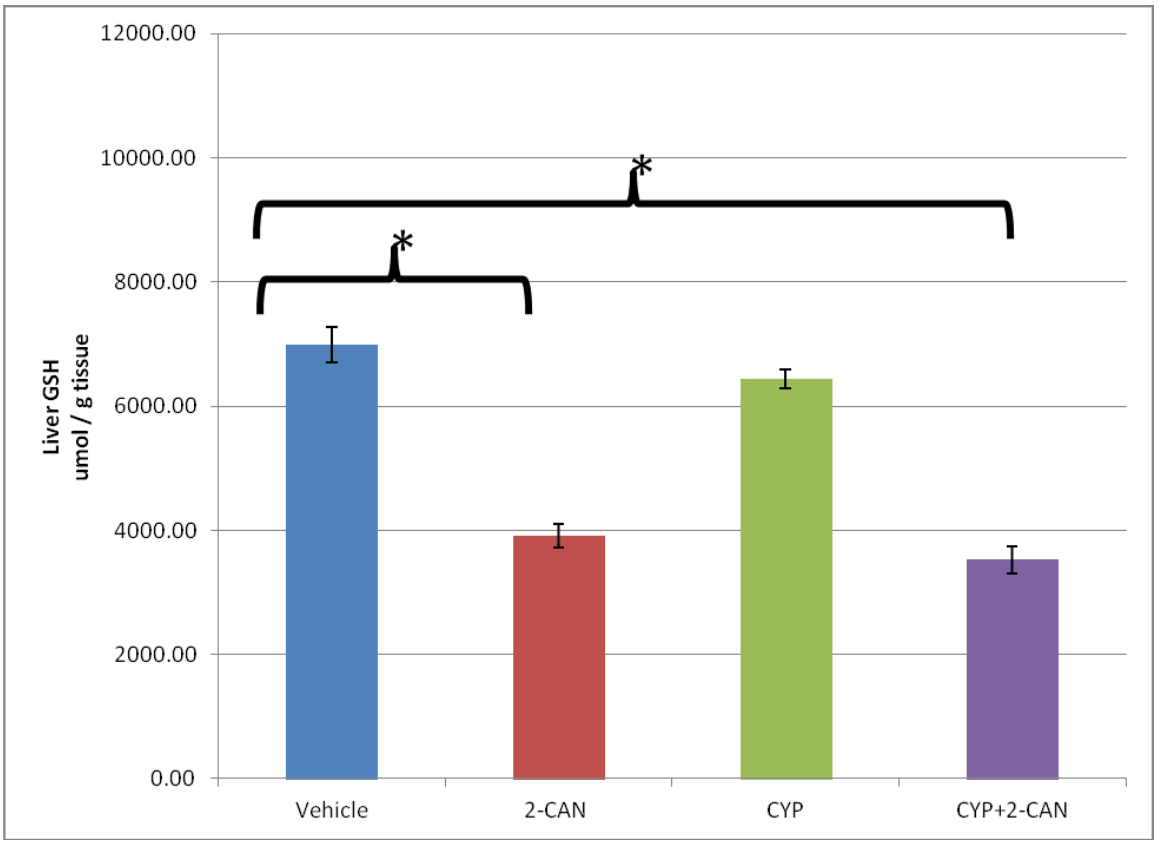


Figure 14. Comparison of GSH levels in the liver of rats administered vehicle (i.p.); 2-CAN (40 mg/kg i.p); a CYP inhibitor (5P1P); or a CYP inhibitor (5P1P) 1 hour prior to 2-CAN (40 mg/kg i.p). Tissue was removed 3 hours after the last injection. Each bar graph represents the mean ± SEM of 9-11 animals performed in duplicate. Data were analyzed by 1-way ANOVA followed by a Newman-Keuls' post-hoc test where significance (*) indicates $p<0.05$

Figure 15. The effect of 2-CAN and 5P1P on liver GSSG levels

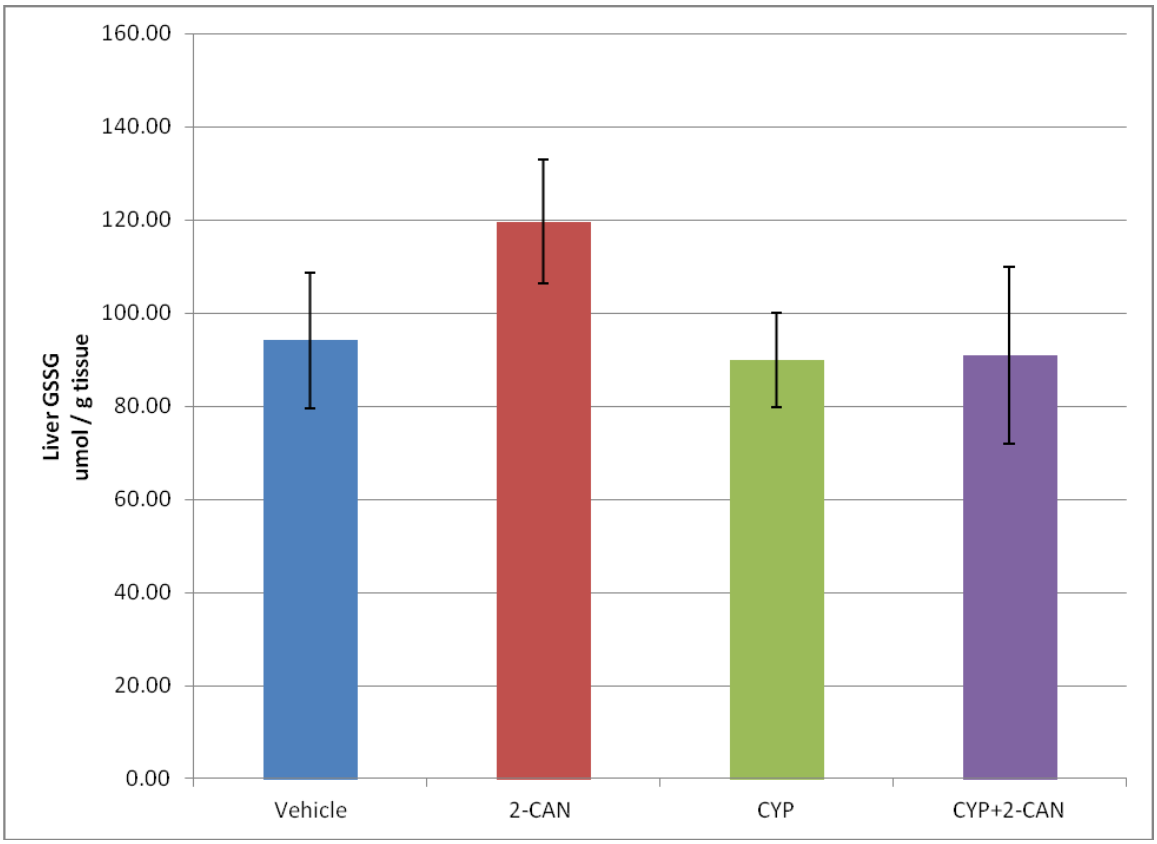


Figure 15. Comparison of GSSG levels in the liver of rats administered vehicle (i.p.); 2-CAN (40 mg/kg i.p); a CYP inhibitor (5P1P); or a CYP inhibitor (5P1P) 1 hour prior to 2-CAN (40 mg/kg i.p). Tissue was removed 3 hours after the last injection. Each bar graph represents the mean ± SEM of 8-10 animals performed in duplicate. Data were analyzed by 1-way ANOVA followed by a Newman-Keuls' post-hoc test. There was no statically significant change between any of the groups.

Figure 16. Relative amounts of GSH and GSSG levels in the liver following 2-CAN and 5P1P administration

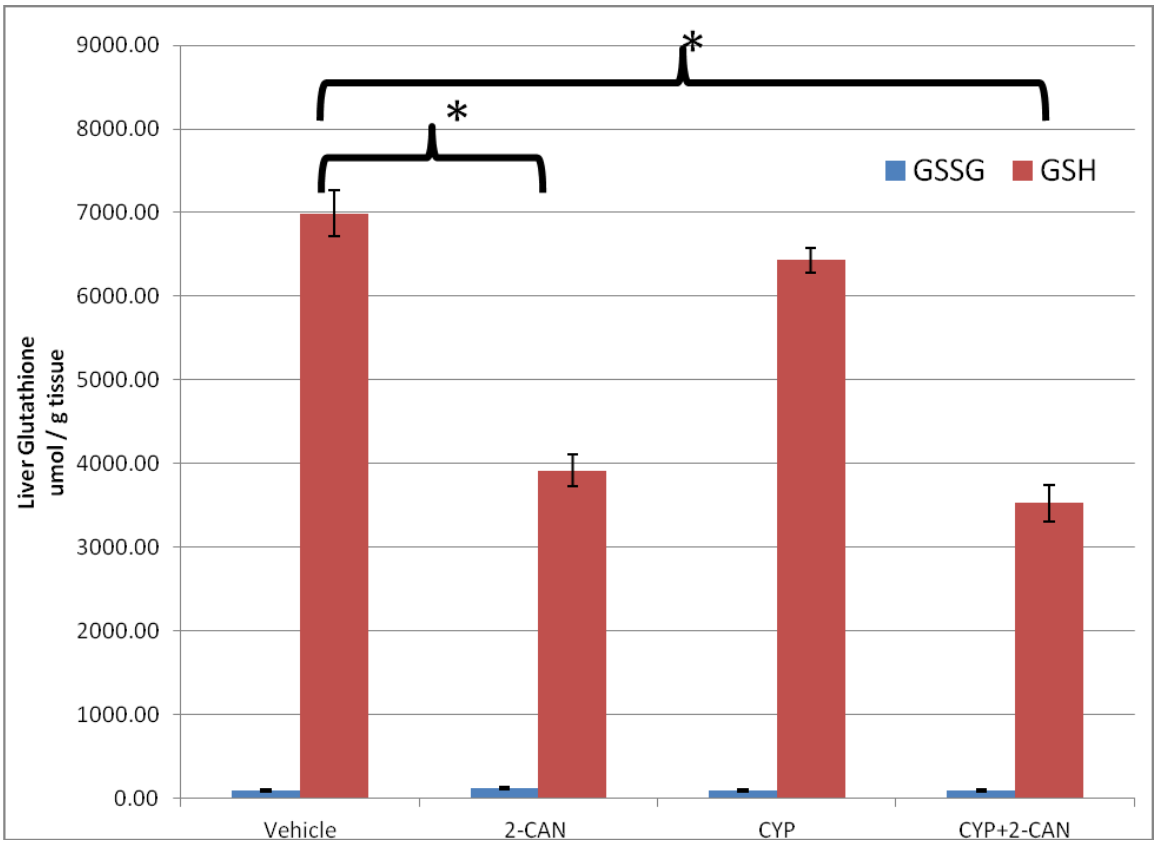


Figure 16. Relative amounts of GSH and GSSG in the liver of rats administered vehicle (i.p.); 2-CAN (40 mg/kg i.p); a CYP inhibitor (5P1P); or a CYP inhibitor (5P1P) 1 hour prior to 2-CAN (40 mg/kg i.p). Tissue was removed 3 hours after the last injection. Each bar graph represents the mean ± SEM of 8-11 animals performed in duplicate. Data were analyzed by 1-way ANOVA followed by a Newman-Keuls' post-hoc test where significance (*) indicates $p<0.05$
* $p<0.05$; n=8-11

Previous work from Mostowy showed that 40 mg/kg of 2-CAN (i.p.) will increase blood cyanide levels, but well below the level known to produce adverse effects. In addition to repeating the work by Mostowy, this study will determine the effect of a CYP inhibitor (5P1P) administered 1 hour prior to 2-CAN. It is expected the animals administered 5P1P prior to 2-CAN would have a reduction in cyanide levels. Based on the proposed toxic metabolic pathway for 2-CAN, cyanide can only be formed after oxidation by CYP2E1. A CYP inhibitor would reduce the amount of oxidation and therefore the amount of cyanide formed. **Figure 17** shows the effect of 2-CAN on the blood cyanide levels with and without the CYP inhibitor 5P1P. The blood cyanide concentration in the 2-CAN group was significantly increased compared to vehicle. Although there appears to be an increase in blood cyanide levels in the 5P1P + 2-CAN group, the increase was not statistically significant compared to the vehicle group.

Due to a lack of cyanide formation, the hypothesis of this dissertation is that the cyanide may be oxidized to cyanate. In addition to determining if cyanate is formed following 2-CAN administration, a CYP inhibitor (5P1P) would decrease the amount of oxidation. According to the proposed toxic mechanism for 2-CAN, cyanide and cyanate are only formed after oxidation of 2-CAN. Therefore it would be expected that 5P1P administered prior to 2-CAN would decrease the concentration of plasma cyanate. **Figure 18** shows the effect of 2-CAN on the plasma cyanate levels with and without the CYP inhibitor 5P1P. The plasma cyanate concentration in the 2-CAN group was significantly increased compared to vehicle. For the 2-CAN group, the plasma cyanate concentration increased from 88.9 nmol/L plasma to 435 nmol/L plasma; this is an increase of about 390%. In the group that received 5P1P 1 hour prior to 2-CAN

administration, the plasma cyanate concentration was significantly less than the animals that received 2-CAN alone. For the 5P1P + 2-CAN group, the plasma cyanate concentration decreased from 435 nmol/L plasma to 194.4 nmol/L plasma; this was a decrease of 55%. The increase in cyanate levels in the 5P1P + 2-CAN group was not statistically significant compared to the vehicle or CYP groups.

Figure 17. The effect of 2-CAN and 5P1P on blood cyanide levels

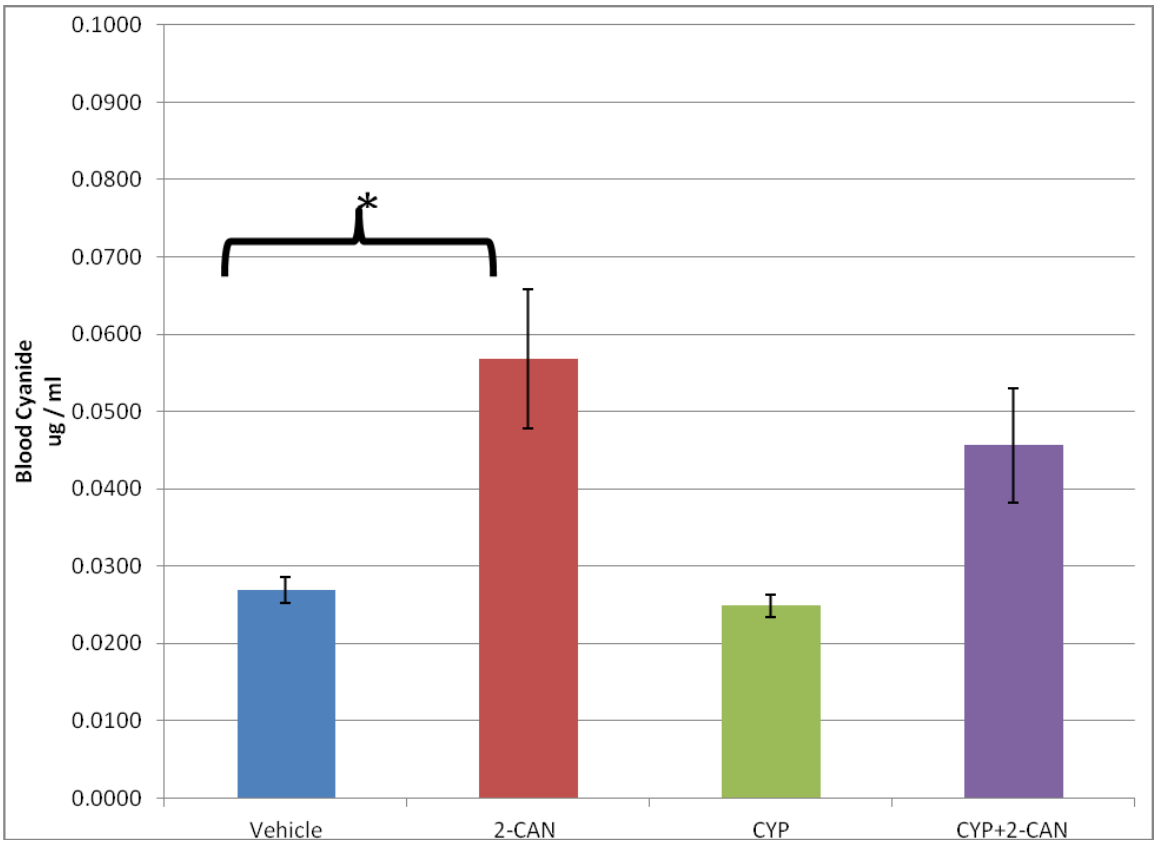


Figure 17. Comparison of blood cyanide levels rats administered vehicle (i.p.); 2-CAN (40 mg/kg i.p); a CYP inhibitor (5P1P); or a CYP inhibitor (5P1P) 1 hour prior to 2-CAN (40 mg/kg i.p). Tissue was removed 3 hours after the last injection. Each bar graph represents the mean ± SEM of 6-7 animals. Data were analyzed by 1-way ANOVA followed by a Newman-Keuls' post-hoc test where significance (*) indicates $p<0.05$

Figure 18. The effect of 2-CAN and 5P1P on plasma cyanate levels

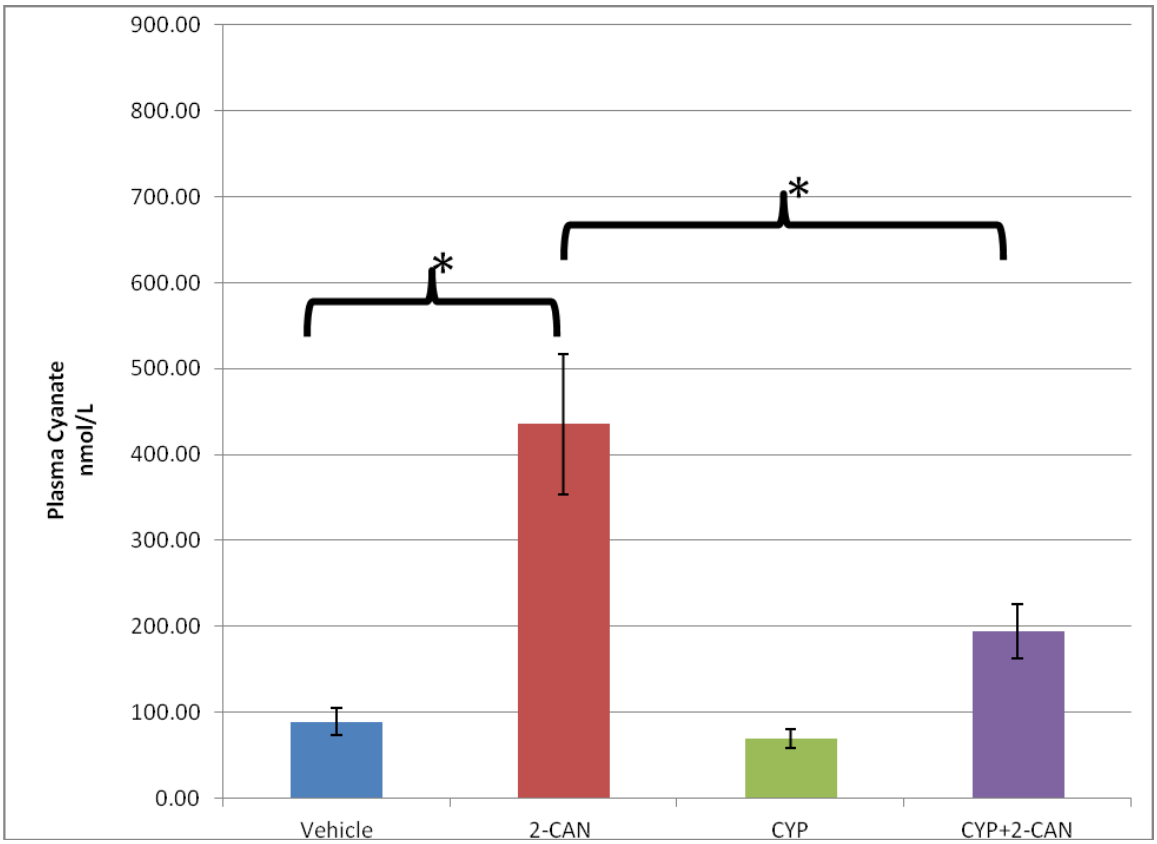


Figure 18. Comparison of plasma cyanate levels rats administered vehicle (i.p.); 2-CAN (40 mg/kg i.p); a CYP inhibitor (5P1P); or a CYP inhibitor (5P1P) 1 hour prior to 2-CAN (40 mg/kg i.p). Tissue was removed 3 hours after the last injection. Each bar graph represents the mean ± SEM of 9-11 animals. Data were analyzed by 1-way ANOVA followed by a Newman-Keuls' post-hoc test where significance (*) indicates $p<0.05$

There is only limited information in the literature associating plasma cyanate concentrations and hind-limb paralysis. To determine if the changes observed with plasma cyanate levels are biologically relevant, **Figure 19** shows the effect of 2-CAN on the time to onset of hind-limb paralysis compared to animals that received 5P1P 1 hour prior to 2-CAN. The time to onset of hind-limb paralysis was significantly increased when 5P1P was administered prior to 2-CAN. The time to onset increased from 6.4 minutes to 16 minutes. This is an increase of 150%.

Figure 19. Time to onset of hind-limb paralysis following 2-CAN and 5P1P administration

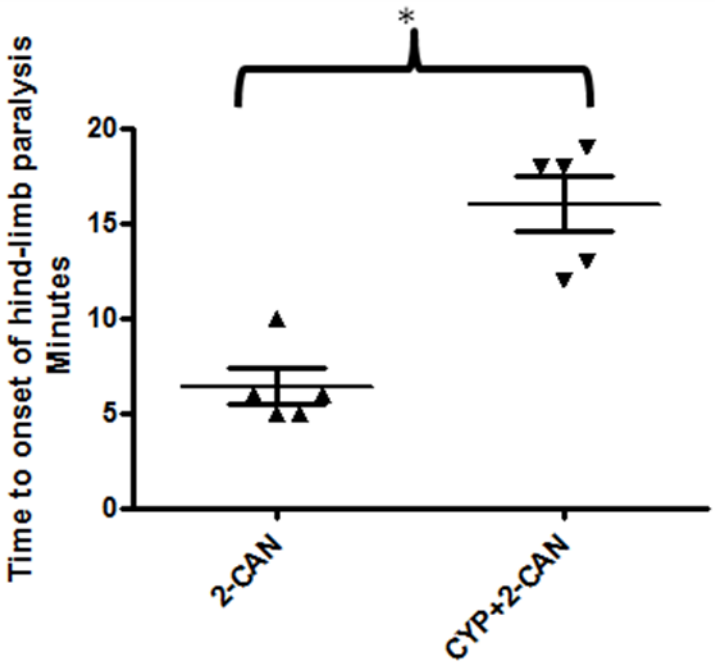


Figure 19.
Comparison of time to onset of hind-limb paralysis in rats administered 2-CAN (40 mg/kg i.p) or a CYP inhibitor (5P1P) 1 hour prior to 2-CAN (40 mg/kg i.p). Shown is a scatter plot of the data where the horizontal line between the points represents the mean data for 5 animals. Each data point (triangles) represents the time for each individual animal. Data were analyzed by an unpaired t-test where significance (*) indicates $p<0.005$

Discussion

The results of this research support the hypothesis that cyanate, not cyanide, is the toxic endpoint for the metabolism of 2-CAN. Three hours after administration of 40 mg/kg 2-CAN, there was a 390% increase in plasma cyanate levels. Blood cyanide levels did increase, but 0.06 µg/L is well below the level known to produce adverse effects. Cyanate is known to cause hind-limb paralysis, but there is little information in the literature if the plasma levels seen following 2-CAN administration (435 nmol/L) are sufficient to induce hind-limb paralysis.

Acrylonitrile (AN) depletes glutathione to a significantly greater extent than 2-chloroacrylonitrile (2-CAN).[100] For example, the LD_{50} for AN is (80 mg/kg (s.c.), and liver GSH levels are decreased by more than 90%.[100] With 2-CAN, the decrease in liver GSH was less than 50%. This indicates that although glutathione depletion is significant, it does not deplete the cells to a point where 2-CAN is covalently binding to tissue protein as observed with AN. It is more likely that 2-CAN is being converted to the epoxide via cytochrome p-450 enzymes or being exhaled unchanged.

Unlike AN which dramatically depletes liver GSH levels, 2-CAN is similar to MAN and only depletes liver GSH by less than 50%. One explanation is that AN is being conjugated and ultimately excreted in the urine via the mercapturic acid pathway. 2-CAN might be metabolized via cytochrome p-450 enzymes or pass through the liver unmetabolized. This pathway is known to occur for MAN which is exhaled unmetabolized in the lungs to a much larger extent then AN.[21] The increased GSH depletion observed with AN could be due its increased reactivity compared to MAN.[100]

Based on the proposed toxic mechanism, cytochrome p-450 activation to the epoxide is needed to form cyanide. If MAN and 2-CAN are excreted unmetabolized, this would explain why they produce less cyanide. Comparing the levels of cyanide formed by AN, MAN, and 2-CAN (**Table 3**) to **Table 2**, the cyanide levels observed following 2-CAN administration are not high enough to be a cause of lethality. One explanation might be the conversion of cyanide to cyanate.

Table 3 – Relative effects of nitrile compounds on GSH depletion and cyanide formation in male Sprague Dawley rats

Chemical	**LD50**	**Dose**	**Route**	**Time**	**Liver GSH depletion (% decrease)**	**Blood Cyanide Level**
AN	80 mg/kg	50 mg/kg	s.c.	1 hour	85%[100]	70 nmol/mL[100]
AN	80 mg/kg	80 mg/kg	s.c.	2 hour	92%[100]	140 nmol/mL[100]
AN	80 mg/kg	115 mg/kg	s.c.	2 hour	93%[100]	160 nmol/mL[100]
MAN	200 mg/kg	100 mg/kg	oral		39%[101]	Not Detectable[46]
2-CAN	20 mg/kg	40 mg/kg	i.p.	3 hour	44%	2 nmol/mL

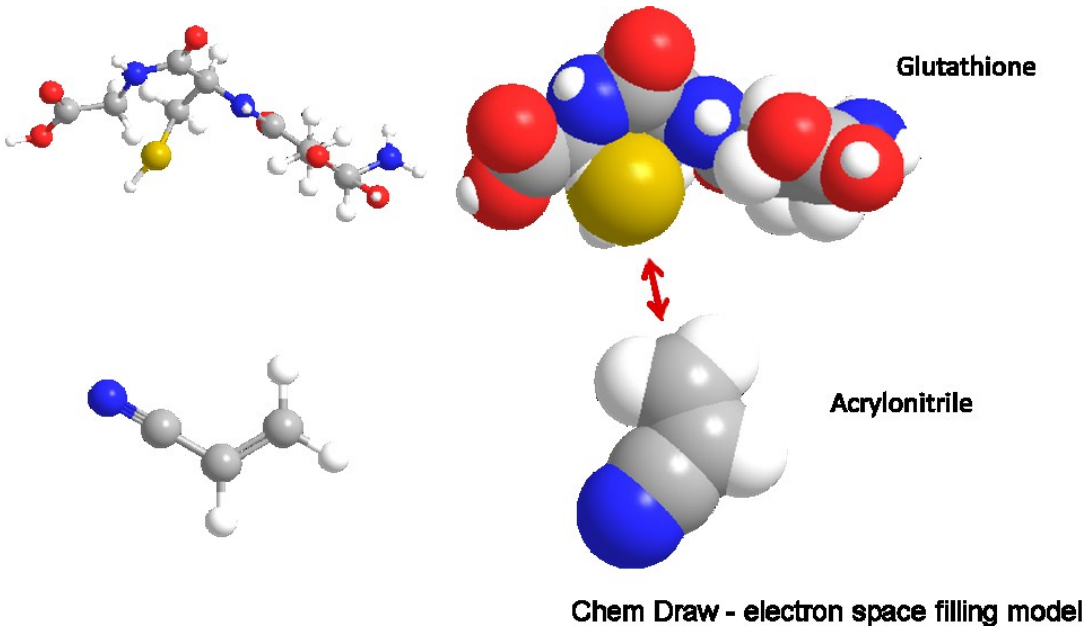
After exposure of the rats to 2-CAN, there was a significant decrease in liver and lung GSH levels after 3 hours. When the animals were pre-treated with 5P1P before 2-CAN exposure, this did not change the GSH depletion. Based on chemical structure and lack of an increase in GSSG levels, it is probable that GSH is directly conjugating with 2-CAN or a 2-CAN epoxide.

Based on the proposed mechanism of 2-CAN (**Figure 20**), it might be expected that 5P1P might cause more GSH depletion by blocking the CYP pathway. The results did not show a change in GSH or GSSG levels after pretreatment with 5P1P. One explanation is that if the glutathione conjugation pathway is saturated and if the CYP pathway is blocked, more 2-CAN will be exhaled unreacted.

N-acetylcysteine (NAC) has been shown to reduce that toxicity of AN in rats by reducing the amount cyanide formed and increasing the LD_{50}.[27] This was thought to happen by providing the sulfur source for the rate limiting step in glutathione synthesis. As described in **Figure 4**., cyanide (CN) is normally metabolized to thiocyanate (SCN^-) via the sulfur-dependent enzyme rhodanese and then excreted in the urine.[27] It may also provide a sulfur source for rhodanese to convert cyanide to thiocyanate for normal metabolism and prevent the formation of cyanate.

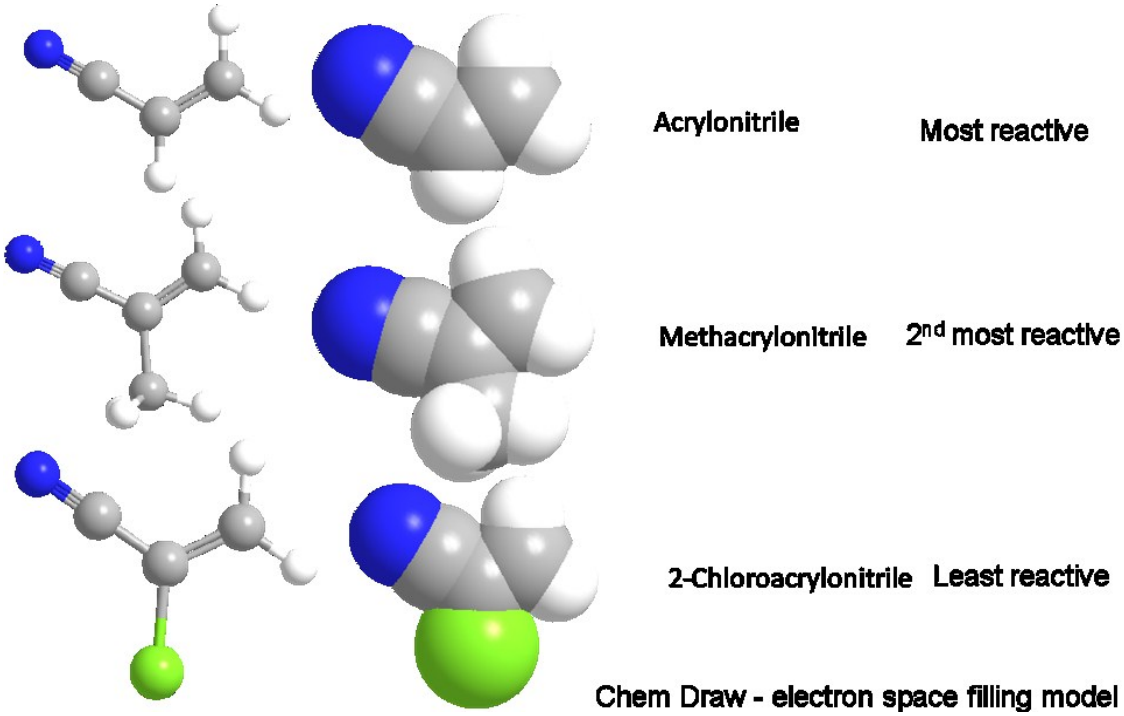
Based on the data obtained, it appears some 2-CAN is metabolized by direct conjugation with GSH, but a portion of 2-CAN is also oxidized to the epoxide. The epoxide can then be metabolized by epoxide hydrolase (EH), reduced, or the epoxide can be conjugated with GSH. Both the EH and reduction pathways could lead to cyanide formation, which was the original hypothesis for the toxic endpoint of 2-CAN. Unfortunately the data generated by this dissertation indicates that free cyanide is not found in the blood. Though blood cyanide levels were increased in the 2-CAN group, levels did not exceed 0.06 μg/mL, which is a concentration well below the minimum for producing any adverse health effects. (Refer to Table 2)

Figure 20 – 3D structure and electron space filling model of GSH and AN



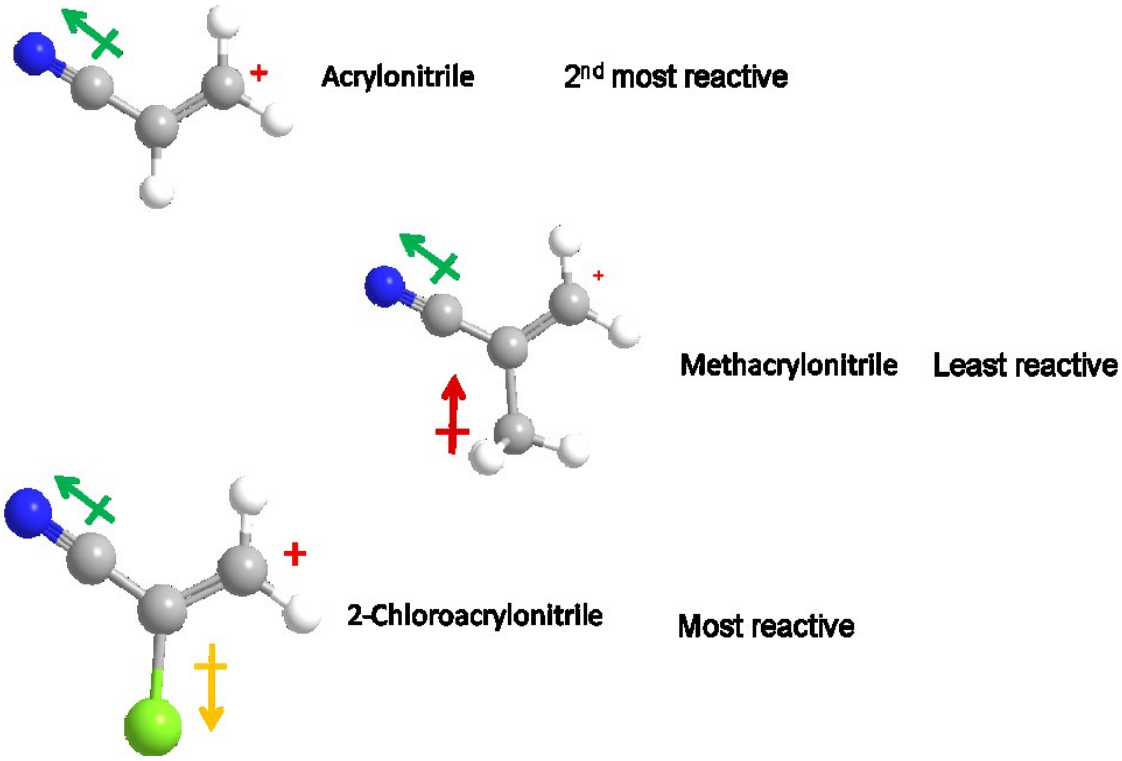
As illustrated by the red arrow in Figure 20, the sulfhydryl group of glutathione must bind with the electron donating position on the nitrile compound.

Figure 21 – Comparison of predicted steric hindrance potential of AN, MAN, and 2-CAN



As illustrated in Figure 21, MAN has more steric hindrance compared to AN. 2-CAN has even more steric hindrance. The larger the compound, the least likely it will be able to bind with GSH. Based on this model, AN is predicted to the most reactive with GSH, followed by MAN, and 2-CAN would be the least reactive.

Figure 22 – Predicted electron donor potential of AN, MAN, and 2-CAN



In addition to physically binding to GSH, the compound must also accept an electron. The larger the partial positive change at the binding site, the more likely it will accept the electron. In the case of AN, MAN, and 2-CAN, all have the same cyanide group creating a partial positive charge. With the addition of the methyl group on MAN, this pulls electrons toward the center of the compound and decreases the partial positive charge. The chlorine group on 2-CAN has the opposite effect of MAN and pulls electrons away from the center of the compound and increases the partial positive charge. Based on electron donating potential, it is predicted that 2-CAN would be the most reactive, followed by AN, and MAN would be the least reactive.

Table 4 – Comparison of predicted electron donor and steric hindrance with actual GSH depletion data.

Compound	**Electron Donor**	**Steric Hindrance**	**Rat Liver GSH depletion**
AN	Intermediate	Least	85% [100]
MAN	Least Reactive	Intermediate	39%[102]
2-CAN	Most Reactive	Greatest	44%

Based on the predicted electron donating potential of AN, MAN, and 2-CAN, it would be expected that 2-CAN would be the most reactive and have the greatest GSH depletion. This does not agree with the actual results which indicate that 2-CAN and MAN only have about one half of the amount of GSH depletion that is seen in AN. Based on the predicted steric hindrance, AN has the least steric hindrance and therefore would be most likely to bind and deplete GSH. This agrees with the results seen in rat experiments. Based on this information, steric hindrance is a better predictor of GSH depletion potential than electron donating potential. This would explain why GSH depletion in 2-CAN is not nearly to the extent seen in AN.

As described in **Figure 4**, normally when cyanide is formed in a tissue, it is reacted with a sulfur donor to form thiocyanate and excreted in the urine. It is known that the conversion of acrylonitrile to cyanide is saturable.[39] The conversion of cyanide to cyanate only happens after the thiocyanate pathway is saturated.[53]

If conversion of cyanide to cyanate needs a sulfur depleted system, why do we not see this effect in AN which cause greater GSH depletion? The answer has to do with the

target organ. An adverse effect associated with both cyanate and 2-CAN is hind-limb paralysis. This is most likely an effect on the nervous system. For example, although AN depletes GSH in the liver by 90%[100], it only depletes GSH in the brain by 40%.[103] If 2-CAN causes glutathione depletion in the brain and/or spinal cord, the cyanide to cyanate oxidation could take place in these organs.

To test the hypothesis that a CYP inhibitor would prevent cyanate formation, 5P1P was administered 1 hour prior to 2-CAN. The inhibitor significantly reduced cyanate formation by 55%. To further confirm that cyanate formed by 2-CAN is associated with the hind-limb paralysis, the time to onset was measured in the 2-CAN vs. 5P1P prior to 2-CAN. The time to onset was delayed from 6 to 16 minutes. Early theories indicated that cyanate hind-limb paralysis was a function of demyelization. More recent studies indicate cyanate can quickly cause hind-limb paralysis by interfering with the function of potassium channels in neurons.[68] Increasing the opening of these channels would result in a decrease in neuronal excitability.

Summary 2-CAN mechanism of action:

The original theory proposed by the manufacturer of 2-CAN is that 2-CAN caused cyanide toxicity in animals and humans. This was disproved by Mostowy in 2000 who demonstrated that GSH depletion was most dramatic in rat liver and kidney. The largest decrease was observed at 3 hours after injection of 2-CAN at 40 mg/kg. (i.p.). Cyanide levels observed were sub-clinical.

The current research finds that cyanate formation, not cyanide is the major toxic metabolite. 5P1P reduced the quantity of cyanate formation. Cyanate levels were biologically relevant because the cyanate decreased by 5P1P caused a delay in the onset

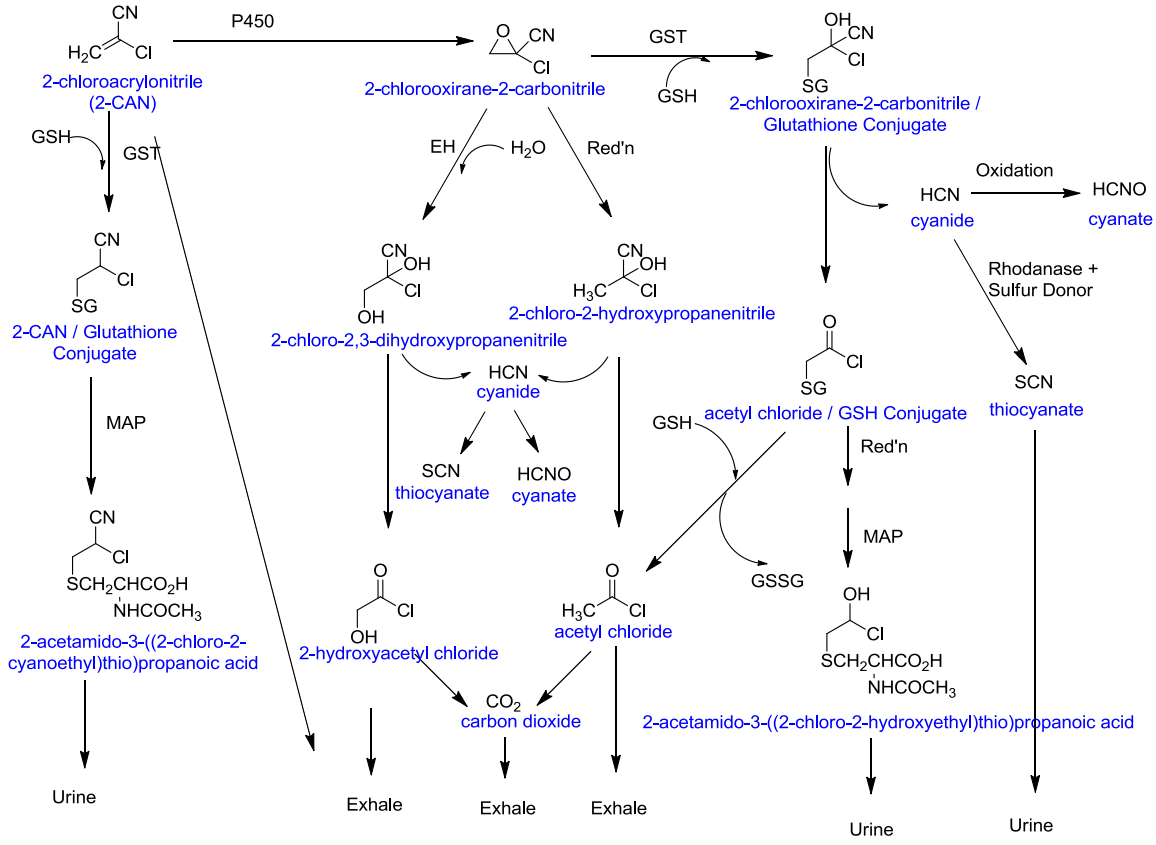
of hind-limb paralysis. However, 5P1P administration did not significantly alter glutathione levels.

Based on the results of this research, Figure 20 describes the proposed mechanism of 2-CAN. The key difference from MAN and AN is the conversion of cyanide to cyanate. This is due to the depletion of sulfur sources as indicated by the reduction in glutathione.

The kinetics of 2-CAN is not known. When CYP2E1 knockout mice were administered MAN orally, the amount of radiolabeled CO_2 (a metabolite of the epoxide formed from cytochrome p-450s) decreased 3-5 fold compared to wild-type.[48] When cyanide was administered orally to rats on a sulfur deficient diet, the plasma cyanate level was 4 fold higher than control animals.[53]

2-CAN was shown to deplete glutathione. Glutathione synthesis is rate limited by sulfur availability. Therefore, it would be expected that cells would be deficient in sulfur. Glutathione depleted cells have less capacity to reduce free radicals. When cyanide is formed following CYP activation to epoxide, it is expected there would not be a sulfur source for normal rhodanese detoxification of cyanide because it requires a sulfur source. In combination with decreased glutathione for reduction, it expected that cyanide would be oxidized to cyanate.

Figure 23 – Proposed Mechanism of 2-CAN



Significance of this research

The current research showed for the first time that 2-CAN administration could cause cyanate formation. With additional testing in rats or possibly humans, suppliers of 2-CAN may be able to remove any recommendation for treatment associated with cyanide toxicity and instead focus on treatment for cyanate toxicity. For example, immediately after exposure a person could be given an antidote to enhance glutathione levels, presumably in the brain or CNS. Often this will be a compound such as NAC. In addition to acting as a precursor to GSH synthesis, it would act as a sulfur source for rhodanese to convert cyanide to thiocyanate.

Conclusion

The results of this research support the hypothesis that acute exposure to 2-CAN causes glutathione depletion, minimal cyanide formation, and cyanate formation. Pre-treatment with a CYP2E1 inhibitor, 5P1P, significantly reduced cyanate formation and the time to onset of hind-limb paralysis.

Blood cyanide levels 3 hours after exposure were too low to cause a toxic effect. When the animals were pre-treated with 5P1P before 2-CAN exposure, there was no change in the trace amount of cyanide formed. This result indicates that with or without a CYP450 inhibitor, cyanide levels were not biologically significant.

Plasma cyanate levels 3 hours after exposure were significantly increased in the 2-CAN exposed group. When administered 5P1P prior to 2-CAN exposure, the amount of cyanate formed was reduced by more than 50%.

Acute onset hind-limb paralysis was observed with animals following 2-CAN exposure. The time to onset of hind limb paralysis was measured in the 2-CAN group and animals that received the CYP inhibitor 5PS1P prior to 2-CAN. It took the inhibitor group an average of 16 minutes to have the first signs of hind-limb paralysis. This is a significantly longer period of time compared to an average of 6 minutes for the 2-CAN only group.

The data in this research indicate that cyanide formation is not the primary toxic intermediate formed from 2-CAN exposure but suggests that cyanate formation plays a significant role in the neurological effect observed with hind limb paralysis. A CYP inhibitor administered prior to 2-CAN exposure can reduce cyanate formation and delay the onset of hind-limb paralysis.

V. Appendices

Appendix A

1. Solutions for cyanate analysis

Cyanate Stock Solution (5 µmol/l)

Dissolve 81.12 mg potassium cyanate in 1 L of de-ionized water to create 1 mM solution. Mix completely. Remove 0.5 mL of this solution (1 mM) and bring to a total volume of 100 mL with de-ionized water.

The shelf life of this solution is short and must be created at the beginning of each experiment.

0.2 mol/L sodium phosphate buffer (pH 7.4)

Create a 1M Sodium Phosphate Monobasic solution by dissolving 12g of sodium phosphate monobasic in 100 mL of water.

Create a 1M Sodium Phosphate Dibasic solution by dissolving 14.2g of sodium phosphate dibasic in 100 mL of water.

Combine 77.4mL of 1M sodium phosphate dibasic and 22.6mL of 1M sodium phosphate monobasic and bring the total volume to 500mL by adding de-ionized water.

4 mol/L acetic acid

Acetic acid is used to adjust the pH to 4.7. This is critical to ensure the proper derivative is formed (See **Figure 9**)

Add 77mL of de-ionized water to a 100mL graduated cylinder. Add 23mL of glacial acetic acid for a total volume of 100mL.

40 mmol/L 2-aminobenzoic acid (adjusted to pH 4.7)

2-aminobenzoic acid reacts with cyanate to form 2-ureidobenzoic acid, the first reaction step in the derivatization process.

Dissolve 556 mg of Anthranilic Acid (2-aminobenzoic acid) in 100 mL of de-ionized water. Place solution in flask with mixing rod. While mixing, enter the

probe for the pH meter and slowly add small amounts of sodium acetate until the pH has been adjusted to 4.7.

The shelf life of this solution is short and must be created at the beginning of each experiment.

6 mol/L sulfuric acid

Sulfuric acid is used to react 2-ureidobenzoic acid to 2,4-quinazolinedione which is ultimately measured in the fluorescence detector.

Add 66.6mL of de-ionized water to a 100mL graduated cylinder. Add 33.3mL of concentrated sulfuric acid (18M) for a total volume of 100mL.

5 mol/L Hydrochloric acid

Hydrochloric acid is used to adjust pH.

Add 57mL of de-ionized water to a 100mL graduated cylinder. Add 43 mL of concentrated HCl (11.6M) for a total volume of 100mL.

3 mol/L Sodium Hydroxide

Sodium hydroxide is used to adjust pH.

Add 70mL of de-ionized water to a 100mL graduated cylinder. Add 30mL of 10M Sodium Hydroxide solution for a total volume of 100mL.

HPLC Mobile Phase for cyanate analysis

Methanol is filtered using a 0.45uM Millipore filter attached to a vacuum flask.

HPLC-grade water is filtered using a 0.45uM Millipore filter attached to a vacuum flask.

It is critical to filter all reagents for mobile phase and use HPLC-grade water to prevent the column from clogging.

1 L of the filtered methanol is then added to 2 L of the filtered water

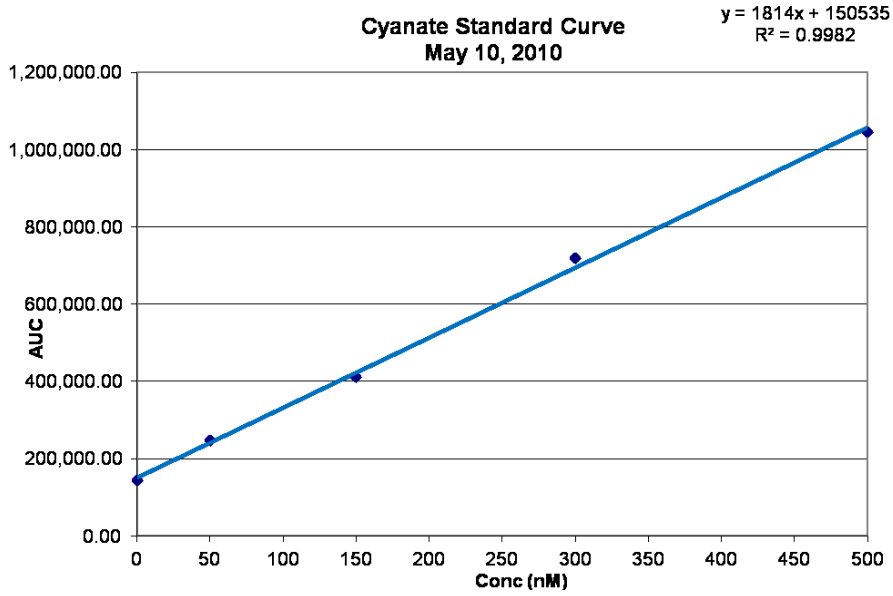
Appendix B

1. Method to prepare the standard curve.

The day of each new experiment, a standard curve was run in parallel to the animal sample. Glass test tubes were labeled and a known amount of the potassium cyanate stock solution was added to each tube. De-ionized water was then added to bring the total volume in each tube to 1 mL.

Volume of cyanate stock solution added to tube	**Total Concentration in tube**
0 μL	Blank
10 μL	50 nM
30 μL	150 nM
60 μL	300 nM
100 μL	500 nM

After the same procedure is followed as with the plasma (with the exception of the Minisart filter) the standard is run through the HPLC with fluorescence detection and the area under the curve (AUC) is recorded and plotted against the known concentrations in each of the tubes. Listed below is an example standard curve.


Cyanate Standard Curve
May 10, 2010
y = 1814x + 150535
R² = 0.9982
1,200,000.00
1,000,000.00
800,000.00
600,000.00
400,000.00
200,000.00
0.00
AUC
0
50
100
150
200
250
300
350
400
450
500
Conc (nM)

Appendix C

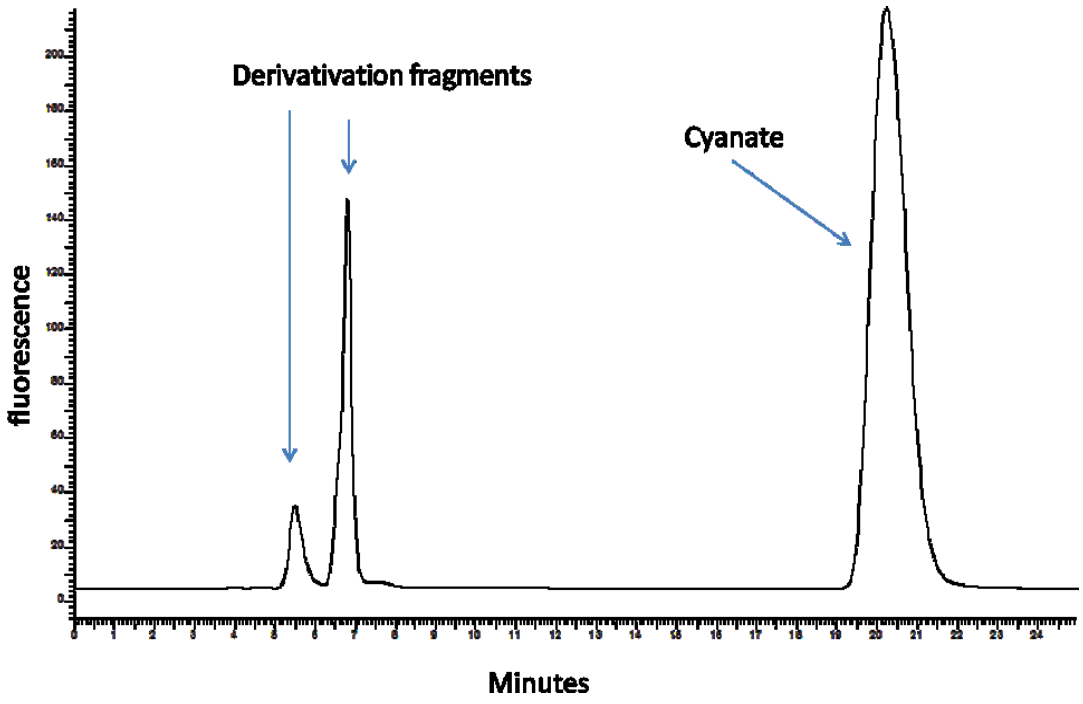
1. Method to analyze on a HPLC

Autosampler

The dried sample is dissolved in 0.5mL of cyanate mobile phase and then added to the HPLC autosampler vial. After being capped, it is loaded into the autosampler.

HPLC setup

The cyanate prefilter (Kromasil C-18 (5µm, 10x3.2 mm); Hicrome Ltd) was added in line prior to the cyanate column (Kromasil KR 100-5C-18 (5µm 250x4.6mm); Hircrom Ltd) . The pump was started and run at a steady rate of 1 mL/min for 30 minutes to prepare the system. The fluorescence detector was set for excitation at 313nm and emission at 353nm. The autosampler was programmed to inject 100µL into the line. The system would record the emission for 30 minutes and then inject the next sample. Based on the standard curve, the peak at approximately 20 minutes is the derivatized potassium cyanate.


Derivatavation fragments
Cyanate
fluorescence
Minutes

Appendix D

1. Calculation to determine the cyanate concentration

The standard curve samples were derivatized and run through the HPLC in the method described previously. The area under the curve (AUC) was determined by the HPLC control software (TurboChrom). The AUC for the cyanate peak was entered into Microsoft Excel and plotted against the known concentrations. The Microsoft Excel slope function was then used to determine the slope (*m*) and the Microsoft Excel intercept function was used to determine the intercept (*b*).

The experiment samples were then derivatized and run through the HPLC in the method described previously. The AUC for the cyanate peak was then entered into Microsoft Excel. Using the straight line equation of $y = mx + b$, the concentration of the experiment sample was able to be determined.

Appendix E

1. Working Solutions for Glutathione Analysis

0.2 mM m-Cresol Purple

m-Cresol is a pH indicator and also functions as a preservative for the GSH.

8.1 mg of m-cresol purple was dissolved in deionized high purity water for a total volume of 100 mL. At room temperature, the solution has a shelf life of 1 month.

IAA / m-cresol solution (100 mM Iodoacetic Acid in 0.2 mM m-cresol purple)

IAA reacts with GSH to form a s-carboxylmethyl derivative which is ultimately fluorescence labeled with FDNB and detected in the fluorescence detector.

20.8 mg of IAA sodium salt was dissolved in 1 mL of 0.2 mM m-cresol purple solution. This solution has a very short shelf life, and was created each day

PCA / BPDS working solution (1.0 mM BPDS in 10:1 PCA solution)

PCA prevents GSH from being oxidized and also to precipitate protein. BPDS is a metal chelator.

14.3 mL of 70% Perchloric Acid (PCA) was added with care to roughly 75 mL of deionized high purity water while frequently stirring. 53.6 mg of Bathophenanthroline disulfonic acid (BPDS) dissolved in the PCA solution. The total volume was brought up to 100 mL with deionized water. At room temperature this solution has a shelf life of up to 1 month.

BPDS Solution (15 mM BPDS in H_2O)

160 mg of BPDS was dissolved deionized water for a total volume of in 20 mL. At room temperature this solution has a shelf life of up to 1 month.

KOH/$KHCO_3$ Solution

The KOH/$KHCO_3$ solution is added to adjust the pH of the solutions.

10 M KOH is made by dissolving 56.1 g of KOH in 100 mL of deionized high purity water.

3 M $KHCO_3$ is made by dissolving 60.1 g $KHCO_3$ in 200 mL of deionized high purity water.

40 mL of 10 M KOH is then combined with 160 mL of 3 M $KHCO_3$. At room temperature this solution has a shelf life of up to 1 month.

FDNB Solution (1% (v/v) 1/fluoro-2,4-dinitrobenzene in HPLC grade ethanol)

FDNB is a fluorescence compound that binds with the IAA-GSH compound (s-carboxylmethyl derivatives) to form n-dinitrophenyl-s-carboxymethyl derivatives which are detected via the fluorescence detector.

0.5 mL of FDNB was added to 49.5 mL of ethanol. This solution can be stored at 4°C in a dark container with foil for up to 1 month.

γ-Glu-Glu stock solution (4.0 mM γ-Glutamyl-Glutamate (γ-Glu-Glu))

γ-Glu-Glu was used as an internal standard. 11.04 mg of γ-Glu-Glu weighed out and dissolved in 9 mL of 15 mM BPDS solution. The total volume of solution is then diluted to 10 mL with deionized water. This solution was stored in aliquots of 50 µL and 100 µL in a microcentrifuge tube at -20°C for up to 6 months.

γ-Glu-Glu working solution

900 µL PCA/BPDS (0.4 mM) is added to 100 µL γ-Glu-Glu (4 mM) stock that was removed from the freezer. Enough should be made for the entire day and mixed to confirm uniformity.

GSH working solution

The working solution is made so that when added to the various tubes, they all receive the same amount of internal standard.

900 µL PCA/BPDS (0.4 mM) is added to 100 µL γ-Glu-Glu (4 mM) stock that was removed from the freezer. Enough should be made for the entire day and mixed to confirm uniformity.

Mobile phase A (4:1 methonol water)

3.2 L HPLC grade methanol (Filtered)

0.8 L HPLC grade water (Filtered)

Acetate Stock Solution

Add 378 mL glacial acetic acid to 122 mL HPLC grade water

While heating and stirring, slowly add 272g sodium acetate trihydrate

Mobile phase B

3.2 L mobile phase A

0.8 L Acetate Stock Solution

Appendix F

1. Method to Prepare GSH and GSSG Standards

Standards

Standard with concentrations of 35, 70, 105, and 140 of GSH and GSSG µL were run in duplicate. Two other standards were created that contained only 70 µL of GSH or only 70 µL of GSSG. This was to confirm GSH was not be oxidized to GSSG during the analysis.

Approximately 1.5 mg GSH was added to a 1.5 mL microcentrifuge tube and kept on ice. The exact weight was noted in the lab notebook. A sufficient amount of BPDS solution was then added to the tube to have a final concentration of 1.23 mg/mL. For example, if 1.5 mg of GSH was added to the tube. 1.5 divided by the MW of GSH (1.23) would be 1.22 mL of BPDS solution to add to make the final. 150 µL of this GSH stock solution is then added to 1350 µL of PCA/BPDS solution in a 2mL microcentrifuge tube for the GSH working solution.

Approximately 3 mg GSSG was added to a 1.5 mL microcentrifuge tube and kept on ice. The exact weight was noted in the lab notebook. A sufficient amount of BPDS solution was then added to the tube to have a final concentration of 2.45 mg/mL. For example, if 3 mg of GSSG was added to the tube. 3 divided by the MW of GSSG (2.45) would be 1.23 mL of BPDS solution to add to make the final. 150 µL of this GSSG stock solution is then added to 1350 µL of PCA/BPDS solution in a 2mL microcentrifuge tube for the GSSG working solution.

PCA/BPA is first added to each tube based on the quantity in the table 5. This was followed by 50 µL of γ-Glu-Glu working solution. Volumes of the GSH working

solution and GSSG working solutions were then added based on the volumes listed in the table 5.

Table 5 – Volumes for glutathione standard curve determination

	GSH Tube	Tube 35	Tube 70	Tube 105	Tube 140	Tube GSSG
PCA/BPDS, µL	380	380	310	240	170	380
γ-Glu-Glu working solution ,µL	50	50	50	50	50	50
GSH working solution, µL	70	35	70	105	140	0
GSSG working solution, µL	0	35	70	105	140	70

The tubes were then mixed on a vortex and 200 µL of the supernatant was removed and placed into a new 1.5 mL microcentrifuge tube.

To each of the supernatant samples, 50 µL of the IAA/m-cresol purple solution was added. The samples were then adjusted to have a pH of 8-9 by using KOH/$KHCO_3$ Solution. The KOH/KHCO3 solution should be added at no more than 50 µL increments to prevent the sample from bubbling over. Typically a total of 130 µL was added to each sample. The solution should turn purple and stay purple after it is vortexed. The samples were the covered in a thick black cloth and put into a laboratory drawer for one hour. The purpose of this step is for the iodoacetic acid to react with the thiols in order to form S-carboxymethyl derivatives. The purpose of the m-cresol purple was not only to function as a pH indicator, but also as a preservative.

The reaction of the iodoacetic acid with the thiols and forming S-carboxymethyl derivatives of thiols eliminated the potential for thiol disulfide interchange. This could have occurred within a few minutes after the cells were disrupter.

An equal volume of FDNB solution was added to each sample (usually 380 µL). They were then incubated in a dark environment at room temperature for 18-24 hours. The tubes were covered by a thick black cloth and put into a lab drawer in order to keep the samples in complete darkness.

The purpose of adding the FDNB was to form 2,4-dinitrophenyl derivatives. At 4°C, these samples were stable for at least two weeks.

After 24 hours in room temperature darkness, the samples were placed in a 13,000X centrifuge for 20 minutes. One hundred µL of the supernatant was added to the autosampler tubes and placed into the autosampler. In addition to samples, a standard curve, GSH only, and GSSG only samples were analyzed via HPLC with UV/Vis dection at a wavelength of 365nm. Each run took about 45 minutes. Using the standard curve, the concentration of GSH and GSSG was calculated for each sample.

Appendix G

1. Glutathione Analysis – High Performance Liquid Chromatography

Solvent and Gradient System

Reverse-phase ion-exchange was used to separate the compounds with a bonded phase packing that contains a bonded surface that was coated with 3-aminopropyl groups. The column used was 20cm x 4.6mm with 5μm exsil silica derivatized with 3-aminopropyltriethoxy-silane purchased from CEL Associates. The excess of 1-fluoro-2,4-dinitrobenzene is rapidly eluted using methanol and appeared as a large peak within the first few minutes of the run. Methanol also eluted excess concentrations of 4-dinitrophenol derivatives of basic and neutral amino acids as well as 2,4-dinitrophenol. In order to maintain the bonded phase amino groups in the protonated form, acetic acid at a pH of 4.5-4.6 was used. This permits selective ion elution of the acidic dinitrophenol derivatives along with the increasing concentration of sodium acetate.

Gradient

The flow rate of the two pumps (one for each mobile phase) was adjusted by a controller to get the following ratios but keep a constant flow of 1.0 mL/min.

0-8 minutes

80% Mobile Phase A

20% Mobile Phase B

8-28 minutes

Start linear gradient moving from current ratio to 100% Mobile phase

28-40 minutes –

Start linear gradient moving from current ratio to 80% A / 20% B

40-45 minutes

80% Mobile Phase A

20% Mobile Phase B

Appendix H

1. Calculations for determining GSH and GSSG in samples

The standards were run in the HPLC using the method described previously. From this, a standard curve was developed which allows for the calculation of the unknown concentrations of GSH and GSSG. The area under the curve (AUC) for the y-glu-glu, GSH, and GSSG peaks were entered into Microsoft Excel and plotted against the known concentrations. The Microsoft Excel slope function was then used to determine the slope (*m*) and the Microsoft Excel intercept function was used to determine the intercept (*b*).

The experiment samples were then derivatized and run through the HPLC in the method described previously. The AUC for the y-glu-glu, GSH, and GSSG peaks were determined by the HPLC control software (TurboChrom). The AUC was then entered into Microsoft Excel. Using the straight line equation of $y = mx + b$, the concentration of the experiment sample was able to be determined

Appendix I

1. HPLC Column Retention Times

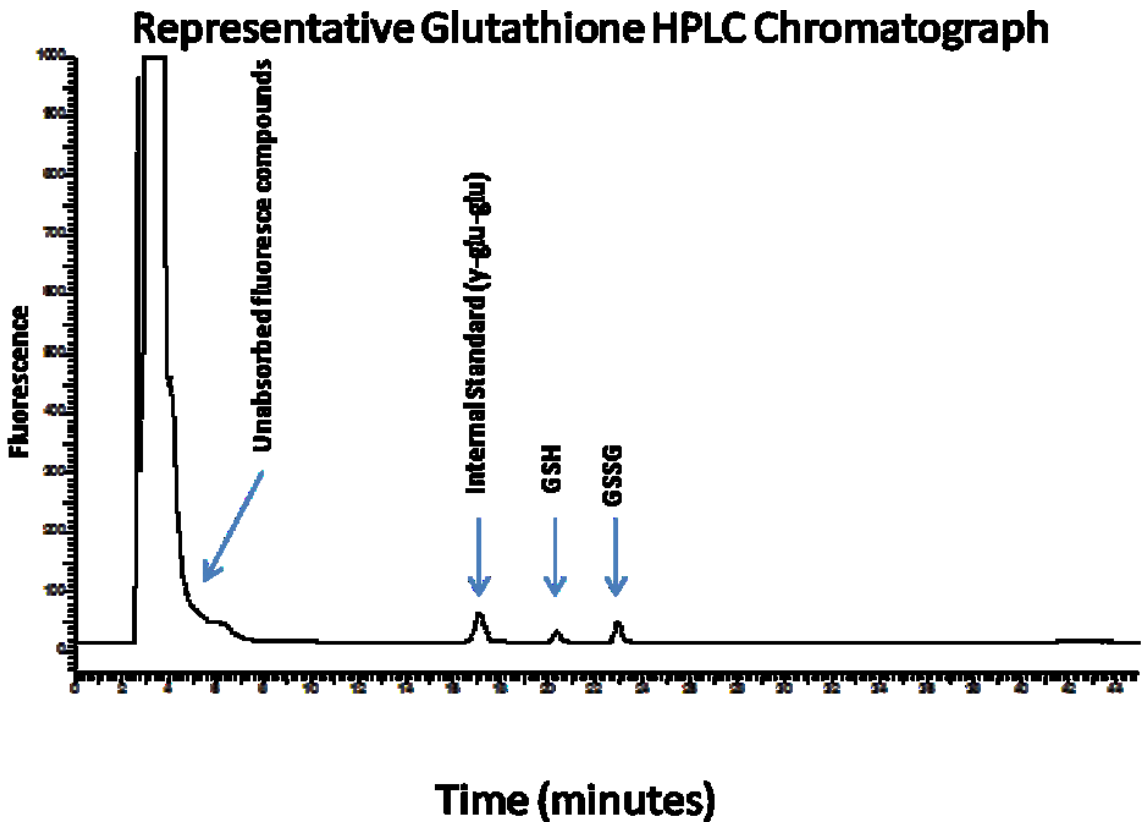
HPLC Chromatograph Retention Times for n-dinitrophenyl-S-carboxymethyl derivatives

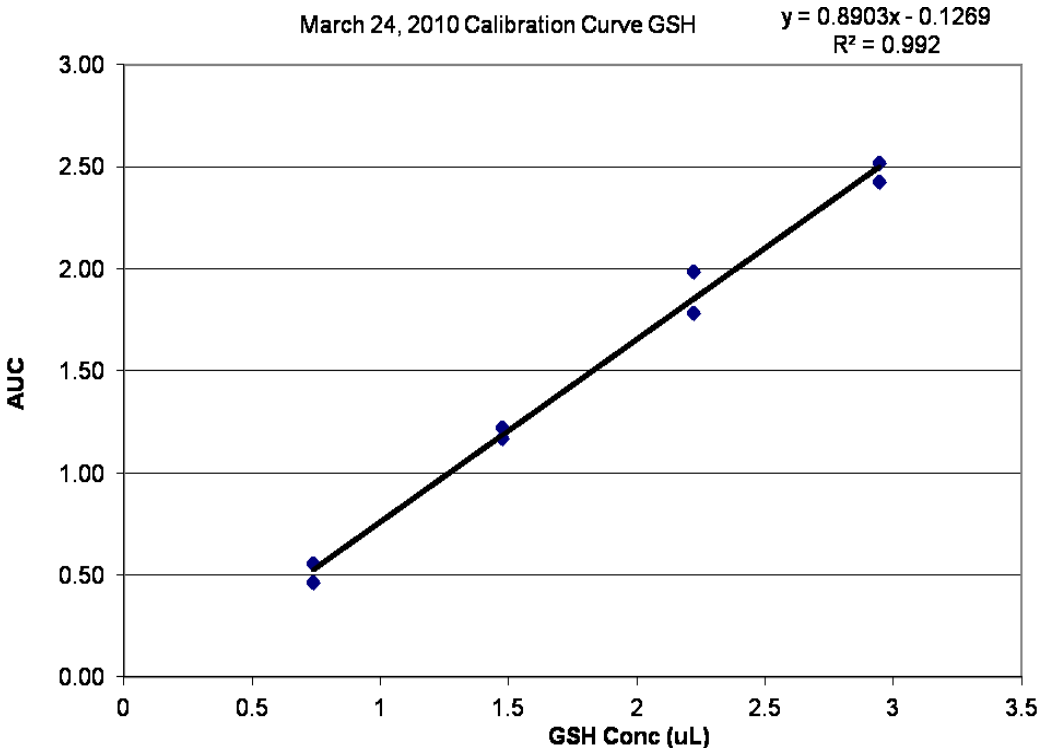
Compound	Retention Time
y-Glutamyl Glutamate (Internal Standard)	15 – 20
Glutathione (GSH)	18 – 22
Glutathione Disulfide (GSSG)	22 - 25

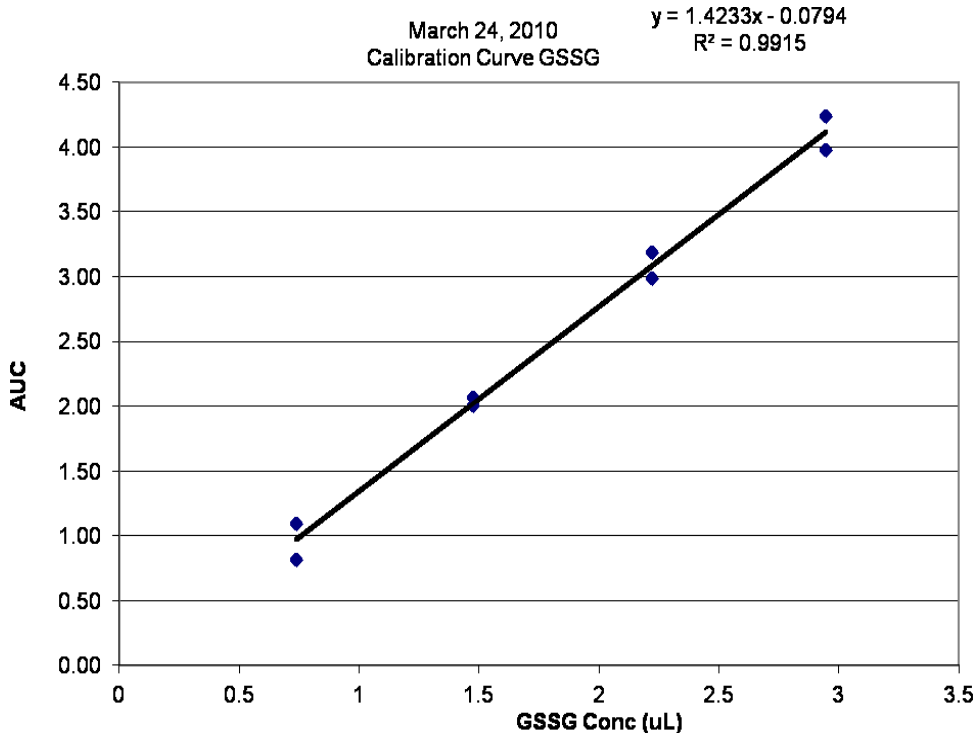
Retention Times will vary depending on the age of the HPLC column.

Appendix J

1. Example HPLC Chromatographs and standard curves




March 24, 2010 Calibration Curve GSH
y = 0.8903x - 0.1269
R² = 0.992
AUC
GSH Conc (uL)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0
0.5
1
1.5
2
2.5
3
3.5


March 24, 2010
Calibration Curve GSSG
y = 1.4233x - 0.0794
R² = 0.9915
AUC
GSSG Conc (uL)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0
0.5
1
1.5
2
2.5
3
3.5

Appendix K

1. Working Solutions for Cyanide Analysis and standard curve

Sodium Hydrogen Phosphate (NaH_2PO_4)

13.8 g NaH_2PO_4 weighed out and placed into a 100 mL volumetric flask. Deionized water was used to fill the volume of the volumetric flask. When refrigerated this solution has a shelf life of up to 1 year.

Chloramine T

125 mg of Chloramine T weighed out and placed into a 50 mL volumetric flask. Deionized water was used to fill the volume of the volumetric flask. When refrigerated this solution has a shelf life of up to 6 months.

Barbituric Acid Reagent

7.5 mL of pyridine was added to 1.5g of barbituric acid in a 25 mL volumetric flask. This solution was mixed and 1.5 mL of concentrated hydrochloric acid (HCl) was added. The solution was mixed and swirled repeatedly. The volume of the volumetric flask was filled and diluted with deionized water. The Solution was mixed frequently over a period of 30 minutes until solution is totally dissolved. When refrigerated this solution has a shelf life of up to 3 months.

0.1 N NaOH

50 mL of 1N NaOH aqueous solution was added to a 500 mL volumetric flask. The volumetric flask was then diluted with deionized water. At room temperature, this solution has a shelf life of up to 6 months.

0.2 N H_2SO_4

2.8 mL of concentrated sulfuric acid was added to a 500 mL volumetric flask. The volumetric flask was diluted with deionized water. At room temperature, this solution has a shelf life of up to 6 months.

10% H_2SO_4

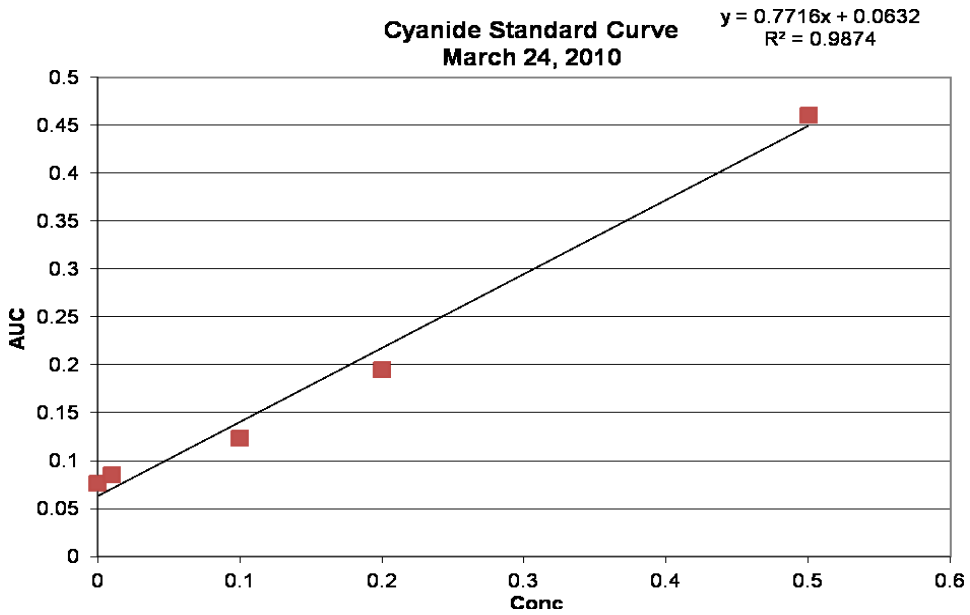
50 mL of concentrated sulfuric acid was added to a 500 mL volumetric flask. The volumetric flash was diluted with deionized water. At room temperature this solution has a shelf life of up to 1 year.

Cyanide Stock Standard

250 mg of potassium cyanide was weighed out. It was then placed into a one liter volumetric flask. The volumetric flask was diluted with 0.1 NaOH. When refrigerated this solution has a shelf life of up to 1 year.

Working Cyanide Standard

1.0 mL of the stock cyanide standard was added to a 100 mL volumetric flask. The volumetric flask was then diluted with 0.1 NaOH. When refrigerated this solution has a shelf life of up to 6 months.



Appendix L – Lung GSH Concentration

Group	Concentration (μmol / g tissue)	Standard Error	Number of animals
Vehicle	1866	88	10
2-CAN	1480*	150	12
CYP	1908	72	9
CYP + 2-CAN	1432*	38	11

* Statistically significant compared to vehicle. $p<0.05$

Appendix M – Lung GSSG Concentration

Group	Concentration (μmol / g tissue)	Standard Error	Number of animals
Vehicle	52	6	7
2-CAN	49	7	8
CYP	85	17	7
CYP + 2-CAN	77	13	11

No statistically significant changes among any of the groups.

Appendix N – Liver GSH Concentration

Group	Concentration (μmol / g tissue)	Standard Error	Number of animals
Vehicle	6989	278	10
2-CAN	3913*	189	10
CYP	6434	149	9
CYP + 2-CAN	3524*	213	11

* Statistically significant compared to vehicle. $p<0.05$

Appendix O – Liver GSSG Concentration

Group	Concentration (μmol / g tissue)	Standard Error	Number of animals
Vehicle	94	15	11
2-CAN	120	13	9
CYP	90	10	8
CYP + 2-CAN	91	20	10

No statistically significant changes among any of the groups.

Appendix P – Blood Cyanide Concentration

Group	Concentration (μg / mL blood)	Standard Error	Number of animals
Vehicle	0.027	0.002	7
2-CAN	0.057*	0.009	7
CYP	0.025	0.002	7
CYP + 2-CAN	0.046	0.007	6

* Statistically significant compared to vehicle. $p<0.05$

Appendix Q – Plasma Cyanate Concentration

Group	Concentration (nmol/L)	Standard Error	Number of animals
Vehicle	88.9	15.7	10
2-CAN	435.7*	81.7	11
CYP	69.3	11.1	9
CYP + 2-CAN	194.4**	31.2	10

* Statistically significant compared to vehicle. $p<0.05$;

** Statistically significant compared to 2-CAN group. $p<0.05$

Appendix R – Time to onset of hind-limb paralysis

Group	Minutes	Standard Error	Number of animals
2-CAN	6.4	0.92	5
CYP + 2-CAN	16*	1.45	5

* Statistically significant compared to 2-CAN group. $p<0.05$

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